ANALYTICAL METHOD DEVELOPMENT AND METHOD VALIDATION OF CINITAPRIDE AND PANTOPRAZOLE IN PHARMACEUTICAL DOSAGE FORMS BY RP-HPLC

Dissertation Submitted to
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In Partial fulfillment for the award of the degree of

MASTER OF PHARMACY

IN

PHARMACEUTICAL ANALYSIS

Submitted By

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This is to certify that the dissertation work entitled **"ANALYTICAL METHOD DEVELOPMENT AND METHOD VALIDATION OF CINITAPRIDE AND PANTOPRAZOLE IN PHARMACEUTICAL DOSAGE FORMS BY RP-HPLC",** submitted by the student bearing **Reg. No: 261430216** to "**The Tamil Nadu Dr.M.G.R.Medical University – Chennai**" , in partial fulfillment for the award of Degree of **Master of Pharmacy** in **Pharmaceutical Analysis** was evaluated by us during the examination held on……..……

Internal Examiner **External Examiner**

CERTIFICATE

This is to certify that the work embodied in this dissertation "**ANALYTICAL METHOD DEVELOPMENT AND METHOD VALIDATION OF CINITAPRIDE AND PANTOPRAZOLE IN PHARMACEUTICAL DOSAGE FORMS BY RP-HPLC",** submitted to The Tamil Nadu Dr.M.G.R.Medical University, Chennai, was carried out by **Mr. D.RAMESH KANNAN [Reg.No: 261430216],** for the Partial fulfillment of degree of **MASTER OF PHARMACY** in **PHARMACEUTICAL ANALYSIS** under my guidance and supervision during the academic year 2015-2016.

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The work presented in this dissertation entitled **"ANALYTICAL METHOD DEVELOPMENT AND METHOD VALIDATION OF CINITAPRIDE AND PANTOPRAZOLE IN PHARMACEUTICAL DOSAGE FORMS BY RP-HPLC",** was carried out by me, under the direct supervision of **Mr. S. JAYASEELAN, M.Pharm, Ph.D,** Assistant Professer of Pharmaceutical Analysis, J.K.K.Nattaraja College of Pharmacy, Komarapalayam.

I further declare that this work is original and this dissertation has not been submitted previously for the award of any other degree, diploma, associate ship and fellowship or any other similar title. The information furnished in this dissertation is genuine to the best of my knowledge.

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Mr. D.RAMESH KANNAN

(261430216)

Dedicated to
Almighty
My Beloved Parents,
&
My Family Members

CONTENTS

LIST OF ABBRAVATIONS

API	Active pharmaceutical Ingredient
CGLP	Current Good Laboratories Practices
CGMP	Current Good Manufactures Practices
CV	Co-efficient of Variation
°c	Degree centigrade
FTIR	Fourier Transmission Infra Red
ICH	International Conference on Harmonization of
K	Capacity factor
LOD	Limit of detection
LOQ	Limit of Quantitation
mg	Milligram
mcg	microgram
mL	Milliliter
mg/mL	Milligram per milliliter
N	Plate number
NLT	Not less than
NMT	Not more than
nm	Nanometer
PDA	Photo Diode Array
RI	Refractive Index
RP-HPLC	Reverse phase High Performance Liquid Chromatography
RS	Resolution
RSD	Relative Standard Deviation
RT	Retention time
SD	Standard Deviation

T	Tailing factor
USP	United States Pharmacopoeia
UV	Ultra violet
v/v	Volume per volume
µg	Micro gram
µg/mL	Microgram per Milliliter
ppm	Parts per million
Fig	Figure
CIN	Cinitapride
PAN	Pantoprazole

INTRODUCTION

Pharmaceutical analysis deals not only with medicaments (drugs and formulations), but also with their precursors i.e. with the raw material whose degree of purity, which in turn decides the quality of medicaments. The quality of a drug is determined, after establishing its authenticity, which is carried by testing its purity and the quality of the pure substance in the drug and its formulations.

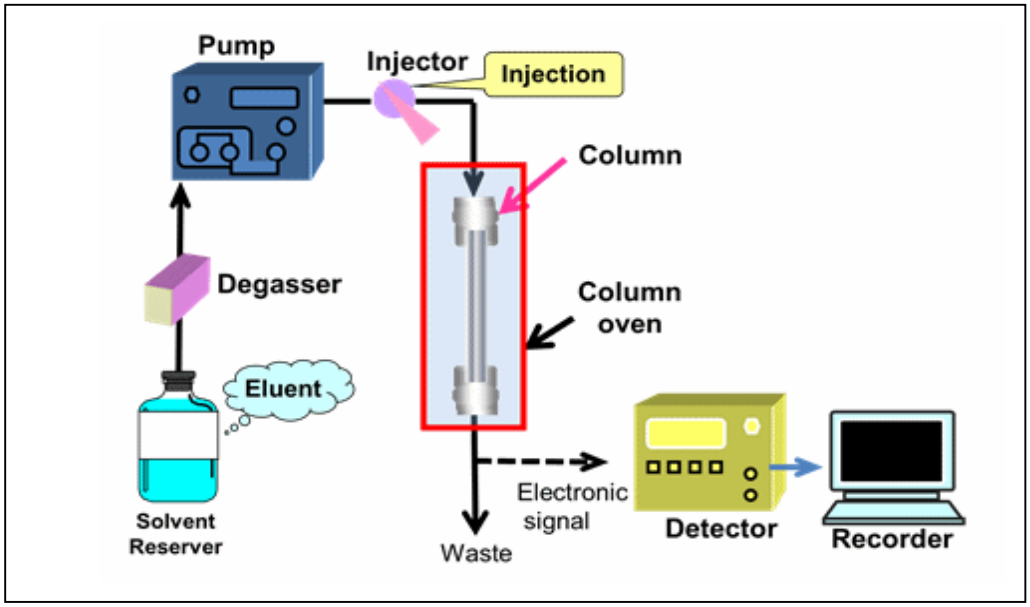
HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC):

High-performance liquid chromatography is a chromatographic technique used to separate the components in a mixture, to identify each component, and to quantify each component .The method involves a liquid sample being passed over a solid adsorbent material packed into a column using a flow of liquid solvent. Each analyte in the sample interacts slightly differently with the adsorbent material, thus retarding the flow of the analytes. If the interaction is weak, and the analytes flow off the column in a short amount of time, and if the interaction is strong, then the elution time is long.

Chromatography may be defined as a method of separating a mixture of components into individual components through equilibrium distribution between two phases**.** (Sharma B.K.1994)

The HPLC method was considered the choice of estimation, since this method is the most powerful of all chromatographic and other separative methods. The HPLC method has enabled analytical chemist to attain great success in solving his analytical problems. The HPLC is the method of choice in the field of analytical chemistry, since this method is specific, robust, linear, precise, and accurate and the limit of detection is low and also it offers the following advantages.

The schematic representation of an HPLC instrument typically includes a sampler, pumps, and a detector. The sampler brings the sample mixture into the mobile phase stream which carries it into the column. The pumps deliver the desired flow and composition of the mobile phase through the column. The detector generates a signal proportional to the amount of sample component emerging from the column, hence allowing for quantitative analysis of the sample components .A digital microprocessor and user software control the HPLC instrument and provide data analysis. Some models of mechanical pumps in a HPLC instrument can mix multiple solvents together in ratios changing in time, generating a composition gradient in the mobile phase. Various detectors are in common use, such as UV/Vis, photodiode array (PDA) or Refractive index (RI).



SCHEMATIC REPRESENTATION OF HPLC SYSTEM

CHROMATOGRAPHY AND ITS TYPES

Chromatography is a family of analytical chemistry techniques for the separation of mixtures. It involves passing the sample, a mixture that contains the analyte, in the "mobile phase", often in a stream of solvent, through the "stationary phase." The stationary phase retards the passage of the components of the sample. When components pass through the system at different rates they become separated in time, like runners in a marathon. Ideally, each component has a characteristic time of passage through the system. This is called its "retention time."

A physical separation method in which the components of a mixture are separated by differences in their distribution between two phases, one of which is stationary (stationary phase) while the other (mobile phase) moves through it in a definite direction. The substances must interact with the stationary phase to be retained and separated by it.

A chromatograph takes a chemical mixture carried by liquid or gas and separates it into its component parts as a result of differential distributions of the solutes as they flow around or over a stationary liquid or solid phase. Various techniques for the separation of complex mixtures rely on the differential affinities of substances for a gas or liquid mobile medium and for a stationary adsorbing medium through which they pass; such as paper, gelatin, or magnesium silicate gel.

Analytical chromatography is used to determine the identity and concentration of molecules in a mixture. Preparative chromatography is used to purify larger quantities of a molecular species.

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THE DIFFERENT TYPES OF CHROMATOGRAPHY

- **Adsorption Chromatography**

Adsorption chromatography is probably one of the oldest types of chromatography around. It utilizes a mobile liquid or gaseous phase that is adsorbed onto the surface of a stationary solid phase. The equilibration between the mobile and stationary phase accounts for the separation of different solutes.

- **Partition Chromatography**

This form of chromatography is based on a thin film formed on the surface of the solid support by a liquid stationary phase. Solute equilibrates between the mobile phase and the stationary liquid.

- **Ion Exchange Chromatography**

In this type of chromatography, the use of a resin (the stationary solid phase) is used to covalently attach anions or cations on it. Solute ions of the opposite charge in the mobile liquid phase are attracted to the resin by electrostatic forces.

- **Molecular Exclusion Chromatography**

Also known as gel permeation or gel filtration, this type of chromatography lacks an attractive interaction between the stationary phase and solute. The liquid or gaseous phase passes through a porous gel, which separates the molecules according to its size. The pores are normally small and exclude the larger solute molecules, but allow smaller molecules to enter the gel, causing them to flow through a larger volume. This causes the larger molecules to pass through the column at a faster rate than the smaller ones.

- **Affinity Chromatography**

This is the most selective type of chromatography employed. It utilizes the specific interaction between one kind of solute molecule and a second molecule that is immobilized on a stationary phase. For example, the immobilized molecule may be an antibody to some specific protein. When this molecule passes solute containing a mixture of proteins, only the specific protein is reacted to this antibody, binding it to the stationary phase.

This protein is later extracted by changing the ionic strength or pH.

Fundamental attitude

In all chromatography there is a mobile phase and a stationary phase. The stationary phase is the phase that doesn't move and the mobile phase is the phase that does move. The mobile phase moves through the stationary phase picking up the compounds to be tested. As the mobile phase continues to travel through the stationary phase it takes the compounds with it. At different points in the stationary phase the different components of the compound are going to be absorbed and are going to stop moving with the mobile phase. This is how the results of any chromatography are gotten, from the point at which the different components of the compound stop moving and separate from the other components.

In paper and thin-layer chromatography the mobile phase is the solvent. The stationary phase in paper chromatography is the strip or piece of paper that is placed in the solvent. In thin-layer chromatography the stationary phase is the thin-layer cell. Both these kinds of chromatography use capillary action to move the solvent through the stationary phase.

TYPES OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

- Based on modes of chromatography
  - Normal phase chromatography
  - Reverse phase chromatography
- Based on principles of separation
  - Adsorption chromatography
  - Ion exchange chromatography
  - Ion pair chromatography
  - Size exclusion chromatography
  - Affinity chromatography
  - Chiral phase chromatography
- Based on elusion technique
  - Isocratic separation
  - Gradient separation
- Based on the scale of operation
  - Analytical HPLC
  - Preparative HPLC

COMPONENTS OF HPLC SYSTEM:

Pump

Pump generates a flow of elute from the solvent reservoir to the system. Most pumps used in current LC system generate the flow by back-and forth motion of a motor –driven piston. (Reciprocating pumps). Because of this piston motion, it produces "pulses". There have been large system improvements to reduce this pulsation and the recent pumps create much less pulse compared to the older ones. Recent analysis requires very high sensitivity to quantify a small amount of analytes, and thus even a minor change in the flow rate can influence the analysis. Therefore, the pumps required for the high sensitivity analysis needs to be highly precise.

Injector

An injector is placed next to the pump. The simplest method is to use a syringe, and the sample is introduced to the flow of eluent. Since the precision of LC measurement is largely affected by the reproducibility of sample injection, the design of injector is an important factor. The most widely used injection method is based on sampling loops. The use of auto sampler (auto-injector) system is also widely used that allows repeated injections in a set scheduled-timing.

Column

The separation is performed inside the column; therefore, it can be said that the column is the heart of an LC system. The packing material generally used is silica or polymer gels. The eluent used for LC varies from acidic to basic solvents. Most column housing is made of stainless steel, since stainless is tolerant towards a large variety of solvents. However, for the analysis of some analytes such as biomolecules and ionic compounds, contact with metals is not desired, thus polyether ether ketone (PEEK) column housing is used instead.

Detector

Separation of analytes is performed inside the column, Whereas a detector is used to observe the obtained separation .The composition of the eluent is consistent when no analyte is present .While the presence of analyte changes the composition of the eluent. What detector does is to measure these differences. This difference is monitored as a form of electronic signal.

On-line detectors

- Refractive index
- UV/Vis Fixed wave length
- UV/Vis variable wave length
- UV/Vis Diode array
- Fluorescence
- Conductivity
- Mass –Spectrometric (LC/MS)
- Evaporative light scattering

Off-line detector

- FTIR spiral disk monitor requires sample transfer on the germanium disk and following scanning in FTIR instrument.

Recorder

The change in eluent detected by a detector is in the form of electronic signal, and thus it is still not visible to our eyes. Nowadays, computer based data processor (integrator) is more common .There are software that are specifically designed for LC system. It provides not only data acquisition, but features like peak-fitting, base line correction, automatic concentration calculation, molecular weight determination, etc.

Degasser

The eluent used for LC analysis may contain gases such as oxygen that are non-visible to our eyes. When gas is present in the eluent, this is detected as a noise and causes unstable baseline. Generally used method includes sparging (bubbling of inert gas), use of aspirator, distillation system, and/or heating and stirring. However, the method is not convenient and also when the solvent is left for a certain time period (e.g., during the long analysis), gas will dissolve back gradually. Degasser uses special polymers membrane tubing to remove gases. The numerous very small pores on the surface of the polymer tube allow the air to go through while preventing any liquid to go though the pore. By placing this tubing under low pressure container, it created pressure differences inside and outside the tubing (higher inside the tubing). This difference let the dissolved gas to move through the pores and remove the gas. Compared to classical batch type degassing, the degasser can be used on-line; it is more convenient and efficient.

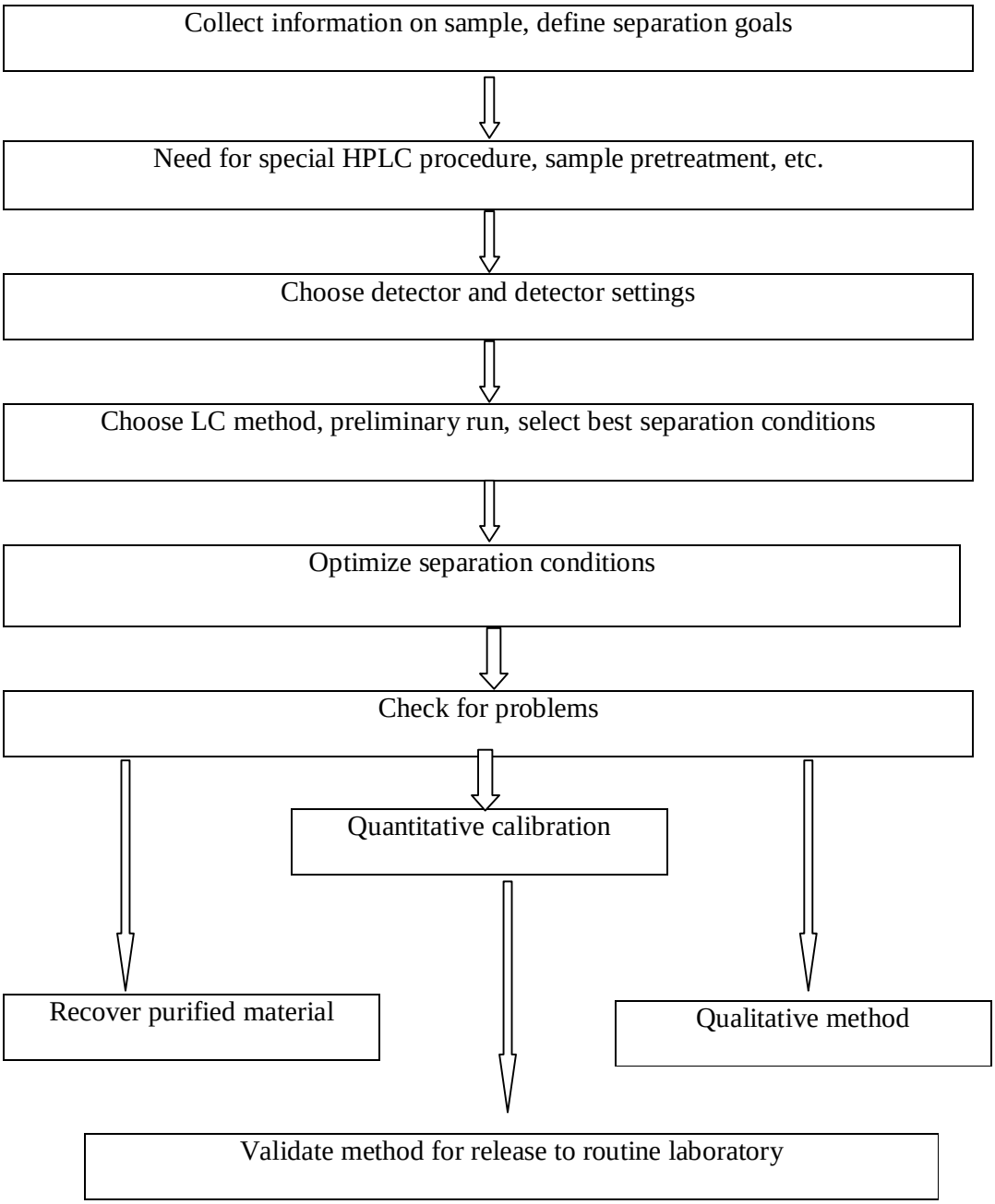
C**olumn heater**

The LC separation is often largely influenced by the column temperature. Also for some analysis, such as sugar and organic acid, better resolutions can be obtained at elevated temperature (50~80^{o}C). It is also important to keep stable temperature to obtained repeatable results even it is analyzed at around room temperature. There are possibilities that small different of temperature causes different separation results. The columns are generally kept inside the column oven (column heater).

(Willard et.al, 1988).

INTRODUCTION TO HPLC METHOD DEVELOPMENT

Method development has following steps:



A good method development strategy should require only as many experimental runs as are necessary to achieve the desired final result. Finally method development should be as simple as possible, and it should allow the use of sophisticated tools such as computer modeling.

The important factors, which are to be taken into account to obtain reliable quantitative analysis, are:

1. Careful sampling and sample preparation.
2. Appropriate choice of the column.
3. Choice of the operating conditions to obtain the adequate resolution of the mixture.
4. Reliable performance of the recording and data handling systems.
5. Suitable integration/peak height measurement technique.
6. The mode of calculation best suited for the purpose.
7. Validation of the development method.

(Synder et.al 1983).

Careful sampling and sample preparation

Before beginning method development, it is need to review what is known about the sample in order to define the goals of separation. The sample related information that is important is summarized in below.

Number of compounds present
Chemical structure
Molecular weight of compounds
pk_a Values of compounds
UV spectra of compounds
Concentration range of compounds in samples of interest
Sample solubility

The chemical composition of the sample can provide valuable clues for the best choice of initial conditions for an HPLC separation.

Separation Goals

The goals of HPLC separation need to be specified clearly, which include:

- The use of HPLC to isolate purified sample components for spectral identification or quantitative analysis.
- It may be necessary to separate all degradants or impurities from a product for reliable content assay.
- In quantitative analysis, the required levels of accuracy and precision should be known (a precision of $\pm$ 1 to 2% is usually achievable).
- Whether a signal HPLC procedure is sufficient for a raw material or one or more different procedure are desired for formulations.
- When the number of samples for analysis at one time is greater than 10, a run time less than 20 minutes often will be important.

Sample preparation

Sample come in various forms:

- Solution ready for injection.
- Solutions that require dilution, buffering, addition of and internal standard or other volumetric manipulation.
- Solids must be dissolved or extracted.
- Samples that require pretreatment to remove interference and /or protect the column or equipment from damage.

Most samples for HPLC analysis require weighing and /or volumetric dilution before injection. Best results are often obtained when the composition of the sample solvents is close to that of the mobile phase since this minimizes baseline upset and other problems. Some samples require a partial separation (pretreatment) prior to HPLC, because of need to remove interference, concentrate sample analyte or eliminate “column killers”.

The samples may be of two types, regular or special. The regular samples are typical mixtures of small molecules (<2000Da) that can be separated by normal starting conditions. Whereas special samples are better separated under customized conditions given below.

Sample	Requirements
Inorganic ions	Detection is primary problem; use ion chromatography.
Isomers	Some isomers can be separated by reserved-phase HPLC and are then classified as regular samples; better separations of isomers are obtained using either
Enantiomers	(1)normal – phase HPLC or(2) reversed –phase separations with cyclodextrin-silica columns. These compounds require “chiral” conditions for their separation.
Biological	Several factors make samples of this kind “special”, molecular conformation, polar functionality, and a wide range of hydrophobicity.
Macromolecules	“Big” molecules require column packing with large pores (>>10-nm diameters); in addition, biological molecules require special conditions as noted above.

Choice of the column

The separation of the column in HPLC is somewhat similar to the selection of columns in G.C, in the sense that, in the adsorption and partition modes, the separation mechanism is based on inductive forces, dipole-dipole interactions and hydrogen bond formation. In case of ion-exchange chromatography, the separation is

based on the differences in the charge, size of the ions generated by the sample molecules and the nature of ionisable group on the stationary phase. In case of size – exclusion chromatography the selection of the column is based on the molecular weight and size of the sample components. Selection of columns based on the method is briefly summarized in below.

Method /Description /Columns	Preferred Method
Reversed – Phase HPLC Uses water- organic mobile phase Columns: C_{18} (ODS), C8, Phenyl, trimethylsilyl (TMS), and cyano.	First choice for most samples, especially neutral or non-ionized compounds that dissolve in water-organic mixtures
Iron –pair HPLC Uses water – organic mobile phase, a buffer to control pH, and an ion –pair reagent Columns: C_{18} , C_8, Cyano	Acceptable choice for ionic or ionisable compounds, especially bases or cations.
Normal-phase HPLC Uses mixtures of organic solvents as mobile phase. Columns: cyano, diol, amino, silica	Good second choice when reserved-phase or ion-pair HPLC is ineffective; first choice for lipophilic samples that do not dissolve well in water-organic mixtures; first choice for mixtures of isomers and for preparative HPLC

Operating conditions to obtain the adequate resolution of the mixture

Most of the drug come under the category of regular samples mean typical mixtures of small molecules (<2000Da) that can be separated using more or less standardized starting conditions. Regular samples can be further classified as neutral or ionic. Samples classified as ionic include acids, bases, amphoteric compounds and organic salts. If the sample is neutral, buffers or additives are generally not required in the mobile phase.

Acids or bases usually require the addition of a buffer to the mobile phase. For basic or cationic samples, less acidic reserve phase columns are recommended. Based on recommendations of the conditions, the first exploratory run is carried and then improved systematically. On the basis of the initial exploratory run isocratic or gradient elution can be selected as most suitable. If typical reverse-phase conditions provided inadequate sample retention, it suggests the use of either ion-pair or normal Phase HPLC. Alternatively, the sample may be strongly retained with 100% ACN as mobile phase suggesting the use of non-aqueous reverse phase chromatography or normal phase HPLC.

Method Development

One approach is to use an isocratic mobile phase of some average organic solvent strength (50%). A better alternative is to use a very strong mobile phase first (80 -100%) then reduce % B as necessary. The initial separation with 100% B results in rapid elution of the entire sample, but few groups will separate. Decreasing the solvent strength shows the rapid separation of all components with a much longer run time, with a broadening of latter bands and reduced retention sensitivity. Goals that are to be achieved in method development are briefly summarized in below.

Goal	Comment
Resolution	Precise and rugged quantitative analysis requires that R_s be greater than 1.5.
Separation time	< 5 – 10 min is desirable for routine procedures.
Quantization	≤ 2% for assay; ≤5% for less-demanding analyses. ≤15% for trace analyses.
Pressure	<150 bar is desirable, <200 bar is usually essential (new column assumed).
Peak height	Narrow peaks are desirable for large signal/noise rations.
Solvent consumption	Minimum mobile – phase use per run is desirable.

Separation or resolution is a primary requirement in quantitative HPLC .The resolution (Rs) value should be maximum (Rs > 1.5) favors maximum precision. Resolution usually degrades during the life of the column and can vary from day to day with minor fluctuations in separation conditions. Therefore, values of Rs =2 or greater should be the goal during method development for sample mixtures. Such resolution will favor both improved assay precision and grater method ruggedness.

Some HPLC assays do not require base line separation of the compounds of interest (qualitative analysis).In such case only enough separation of individual components is required to provide characteristic retention times for peak identification.

The time required for a separation (runtime = retention time for base band) should be as short as possible and the total time spent on method development is reasonable (runtimes 5 to 10 minutes are desirable).

Condition for the final HPLC method should be selected so that the operating pressure with a new column does not exceed 170 bar (2500psi) and upper pressure limit below 2000 psi is desirable. There are two reasons for this pressure limit, despite the fact that most HPLC equipment can be operated at much higher pressures. First, during the life of a column, the backpressure may rise by a factor of as much as due to the gradual plugging of the column by particulate matter. Second, at lower pressure (<170 bars) pumps, sample valves and especially auto samplers operate much better, seals last longer, columns tend to plug less and system reliability is significantly improved. For these reasons, a target pressure of less than 50% of the maximum capability of the pump is desirable, when dealing with more challenging samples or if the goals of separation are particularly stringent, a large number of method development runs may be required to achieve acceptable separation.

(Skoog et.al, 2009)

Repeatable separation

As the experimental runs described above are being carried out, it is important to confirm that each chromatogram can be repeated. When we change conditions (mobile phase, column, and temperature) between method development experiments, enough time must elapse for the column to come into with the new mobile phase and temperature. Usually column equilibration is achieved after passage of 10 to 20 volumes of the new mobile phase through the column. However; this should be confirmed by repeating the experiment under the same conditions. When constant retention times are observed in two such back – to- back repeat experiments ($\pm$ 0.5% or better), it can be assumed that the column is equilibrated and the experiments are repeatable.

Optimization of HPLC method

During the optimization stage, the initial sets of conditions that have evolved from the first stages of development are improved or maximized in terms of resolution and peak shape, plate counts asymmetry, capacity factor, elution time, detection limits, limit of quantitation and overall ability to quantify the specific analyte of interest. Optimization of a method can follow either of two general approaches:

- Manual
- Computer driven

The manual approach involves varying one experimental variable at a time, while holding all other constant and recording changes in response. The variables might include flow rate, mobile or stationary phase composition, temperature, detection wavelength and p^H. This approach to system is slow, time consuming and potentially expensive. However, it may provide a much better understanding of the principles and theory involved and of interactions of the variables.

In the second approach, computer driven automated method development, efficiency is optimized while experimental input is minimized. This approach reduce the time, energy and cost of all instrumental method development.

The various parameters that include to be optimized during method development are

A. Selection of mode of separation.
B. Selection of stationary phase.
C. Selection of mobile phase.
D. Selection of detector.

Selection of mode of separation

In reverse phase mode, the mobile phase is comparatively more polar than the stationary phase. For the separation of polar or moderately polar compounds, the most preferred mode is reverse phase. The nature of the analyte is the primary factor in the selection of the mode of separation. A second factor is the nature of the matrix.

Selection of stationary phase

Selection of the column is the first and the most important step in method development. The appropriate choice of separation column indicates three different approaches.

- Selection of separation
- The particle size and nature of the column packing
- The physical parameters of the column i.e. the length and the diameter some of the important parameters considered while selecting chromatographic columns are
- Length and diameter of the column
- Packing material
- Shape of the particles
- Size of the particles
- % of carbon loading
- Pore volume
- Surface area
- Reproducibility and reliability
- End capping

In this case, the column selected had a particle size of 5µm and an internal diameter of 4.6mm.The column is selected depending on the nature of the solute and

the information about the analyte. Reversed phase mode of chromatography facilities a wide range of columns like dimethyl silane (C_2), butysilane (C_4), octylsilane (C_8), Octadecyslane (C_{18}), base deactivated silane **(**C_{18}), BDS phenyl, Cyanopropyl (CN), nitro, amino etc. silica based columns with different cross linking's in the increasing order of polarity are as follows:

<……..Non-polar…….moderately polar……..polar……..>

$C_{18} < C_8 < C_6 <$ Phenyl < Amino < Cyano < Silica

C_{18} was chosen for this study since it is most retentive one. The sample manipulation becomes easier with this type of column. Generally longer columns provide better separation due to higher the theoretical plate numbers. Columns with 5µm particle size give the best compromise of efficiency.

Peak shape is equally important in method development. Columns that provide symmetrical peaks are always preferred while peaks with poor asymmetry can result in,

- Inaccurate plate number and resolution measurement
- Imprecise quantitation
- Degraded and undetected minor bands in the peaks tail
- Poor retention reproducibility

A useful and practical measurement of peak shape is peak asymmetry factor and peak tailing factor. Peak asymmetry is measured at 10% of full peak height and peak tailing factor at 5%. Reproducibility of retention times and capacity factor is important for developing a rugged and repeatable method.

A column which gives separation of all the impurities and degradants from each other and from analyte peak and which is rugged for variation in mobile phase shall be selected.

Selection of mobile phase

The primary objective in selection and optimization of mobile phase is to achieve optimum separation of all the individual impurities and degradants from each other and from analyte peak.

In liquid chromatography, the solute retention is governed by the solute distribution factor, which reflects the different interactions of the solute – stationary phase, solute-mobile phase, and mobile phase-stationary phase. For a given stationary phase, the nature and the composition of which has to be judiciously selected in order to get appropriate and required solute retention. The mobile phase has to be adapted in terms of elution strength (solute retention) and solvent selectivity (solute separation).Solvent polarity is the key word in the chromatographic separations since a polar mobile phase will give rise to low solute retention in normal phase and high solute retention in reverse phase LC. The selectivity will be particularly altered if the buffer pH is close to the pKa of the analytes. The following are the parameters, which shall be taken into consideration while selecting and optimizing the mobile phase.

- Buffer
- pH of the buffer
- Mobile phase composition

Buffers if any and its strength

Buffer and its strength play an important role in deciding the peak symmetries and separations. Some of the most commonly employed buffers are

- Phosphate buffers prepared using salts like KH_2PO_4, K_2HPO_4, NaH_2PO_4, and Na2HPO4.
- Phosphoric acid buffers prepared using H_3PO_4.
- Acetate buffers-ammonium acetate, sodium acetate etc.
- Acetic acid buffers prepared using CH_2COOH.

The retention also depends on the molar strengths of the buffer-molar strength is increasingly proportional to retention times. The strength of the buffer can be increasing, if necessary to achieve the required separations. The solvent strength is a measure of its ability to pull analyte from the column. It is generally controlled by the concentration of the solvent with the highest strength. The useful pH range for columns is 2 to 8, since siloxane linkages are cleaved below pH-2 while at pH values above eight, silica may dissolve.

Mobile phase composition

Most chromatographic separations can be achieved by choosing the optimum mobile phase composition. This is due to the fact that fairly large amount of selectively can be achieved by choosing the qualitative and quantitative composition of aqueous and organic portions. Most widely used solvents in reverse phase chromatography are methanol and Acetonitrile. Experiments should be conducted with mobile phases having buffers with different pH and different organic phases to check for the best separations of analyte peak. A mobile phase which gives separation of analyte peak

and which is rugged for variation of both aqueous and organic phase by at least $\pm$ 0.2% of the selected mobile phase composition should be used.

Selection of Detector

The detector was chosen depending upon some characteristic property of the analyte like UV absorbance, florescence, conductance, oxidation, reduction etc. The characteristics that are to be fulfilled by a detector to be used in HPLC determination are,

- High sensitivity facilitating trace analysis
- Negligible baseline noise to facilitate lower detection.
- Large linear dynamic range.
- Low dead volume.
- Inexpensive to purchase and operate.

Pharmaceutical ingredients do not absorb all UV light equally, so that selection of detection wavelength is important. An understanding of the UV light absorptive properties of the organic impurities and the active pharmaceutical ingredient is very helpful. For the greatest sensitivity ƛmax should be used. Ultra violet wavelengths below 200nm should be avoided because detector noise increases in this region. Higher wave lengths give greater selectivity.

Performance calculations

Carrying out system suitability experiment does the performance calculations. System suitability experiments can be defined as tests to ensure that the method can generate results of acceptable accuracy and precision. The requirements for system suitability are usually developed after method development and validations have been completed. The criteria selected will be based on the actual performance of the method as determined during its validation. For example, if sample retention times form part of the system suitability criteria, their variation SD can be determined during validation.

System suitability might then require that retention times fall within a $\pm$ 3 SD range during routine performance of the method.

The USP (2000) defines parameters that can be used to determine system suitability prior to analysis include plate number(n),tailing factor(T),resolution(Rs) and relative standard deviation (RSD) of peak height or peak area for respective injections.

The RSD of peak height or area of five injections of a standard solution is normally accepted as one of the standard criteria .For assay method of a major component, the RSD should typically be less than 1% for these five respective injections.

The plate number and / or tailing factor are used if the run contains only one peak. For chromatographic separations with more than one peak, such as an internal standard assay or an impurity method expected to contain many peaks, some measure of separations such as Rs is recommended. Reproducibility of t_R or k value for a specific compound also defines system performance.

The column performance can be defined in terms of column plate number. As the plate count is more the column is more efficient. (Lyoyd.R et.al 1997)

METHOD VALIDATION

The word "Validation'' means "Assessment'' of validity or action of proving effectiveness.

Definition

ICH defines validation as "establish the documented evidence which provides a high degree of assurance that a specific process will consistently produce a product of predetermined specifications and quantity attributes.''

Method validation is the process to confirm that the analytical procedure employed for a specific test is suitable for its intended use. Method need to be validated or revalidated.

- Before their introduction into routine use
- Whenever the conditions change for which the method has been validated, e.g., instrument with different characteristics
- Whenever the method is changed, and the change is outside the original scope of the method.

Purpose of validation

- Enable the scientists to communication scientifically and effectively on technical matter.
- Setting the standards of evaluation procedures for checking compliance and taking remedial action.
- Economic: Reduction in cost associated with process sampling and testing.
- As quality of the product cannot always be assured by routine quality control because of testing of statistically insignificant number of samples.
- Retrospective validation is useful for trend comparison of results compliance to CGMP/CGLP.

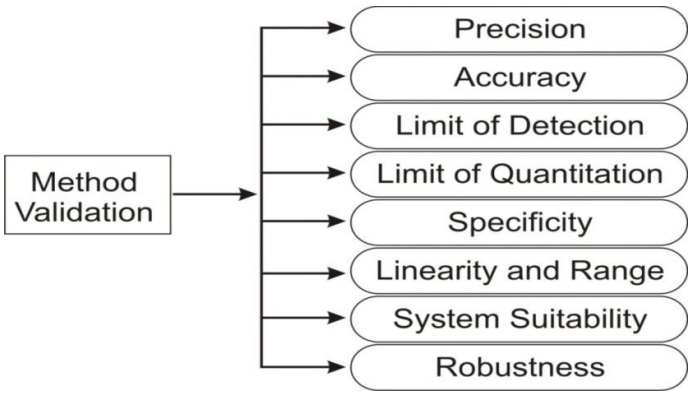
- Closure interaction with pharmacopoeial forum to address analytical problems.
- International pharmacopoeial harmonization particularly in respect of impurities determination and their limits.

Method validation is completed to ensure that an analytical methodology is accurate, specific, reproducible and rugged over the specified range that an analyte will be analyzed. Method validation provides an assurance of reliability during normal use, and is sometime referred to as "the process of providing documented evidence that the method does what it is intended to do''.

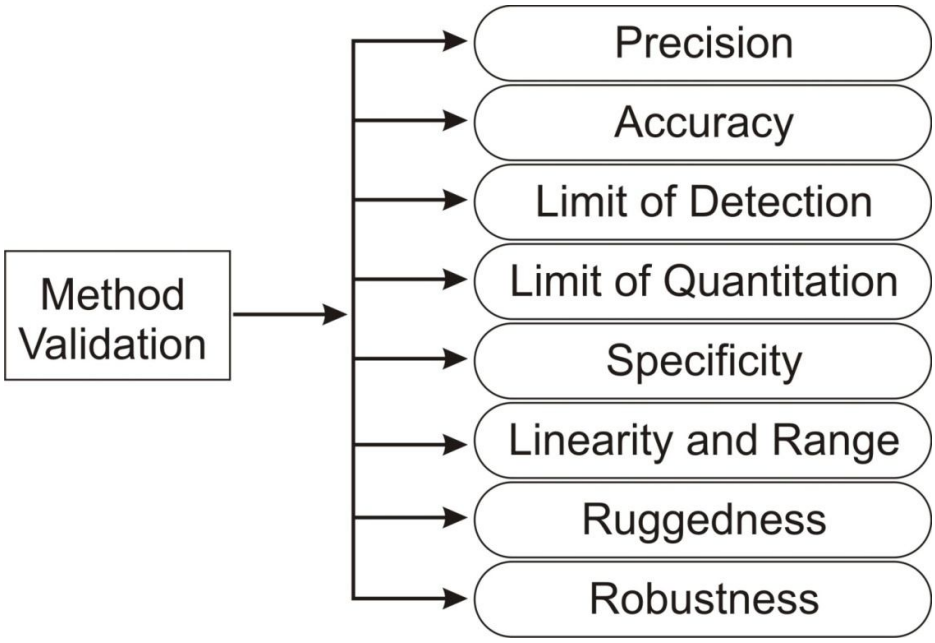
For method validation, these specifications are listed in USP chapter <1225>,and can be referred to as the "eight steps of method validation". as shown in figure below.

These terms are referred to as "analytical performance parameters''. or sometimes as "analytical figures of merit.''

In response to this situation, one of the first harmonization projects taken up by the ICH was the development of a guideline on the "Validation of Analytical Methods" Definitions and Terminology. "ICH divided the "Validation characteristics" somewhat differently, as outlined in Figure below



The USP Eight steps of method validation



ICH Method Validation Parameters

Method validation parameters

The developed methods were validated by following steps:

A. **Accuracy**

It is defined as closeness of agreement between the actual (true) value and mean analytical value obtained by applying a test method number of times. Spike and recovery studies are performed to measure accuracy; a known sample is added to the excipients and the actual drug value is compared to the value found by the assay. Accuracy is expressed as the bias or the % error between the observed value and the true value (assay value/actual value x 100 %.)

The accuracy is acceptable if the difference between the true value and mean measured value does not exceed the RSD values obtained for repeatability of the method. The parameter provides information about the recovery of the drug from sample and effect of matrix, as recoveries are likely to be excessive as well as deficient.

Procedure:

Use a minimum of 3 spiking concentrations in the excipients solution. Prepare two samples of each concentration. Test the 6 samples in triplicate on one run. Measure expected vs. average measured value. Calculate the% recovery.

B. Precision

The precision of an analytical method is the degree of agreement among individual test results when the method is applied repeatedly to multiple sampling of homogenous sample.

Precision is the measure of the degree of repeatability of an analytical method under normal operation and is normally expressed as the percent relative standard deviation (%RSD) or coefficient of variation (% CV) for a statistically significant number of samples. According to the ICH, precision should be performed at three different levels**:** repeatability, intermediate precision, and reproducibility.

Repeatability is the result of the method operating over a short time interval under the same conditions (or) is the % RSD of multiple determinations of a single sample in a single test run (intra-assay precision). It should be determined from a minimum of nine determinations covering the specified range of the procedure (for example, three levels three repetitions each) or from a minimum of six determinations at 100% of the test or target concentration.

Procedure:

- Prepare three dilution of the sample (high/medium/low concentrations in the range).
- Test 10 replicates of each dilution of the sample.
- Calculate the average and standard deviation for each point on the curve.
- Calculate the RSD for each point on the curve.

Intermediate precision is the results from within lab variations due to random events such as different days, analysts, equipment, etc. In determining intermediate precision, experimental design should be employed so that the effects (if any) of the

individual variables can be monitored (or) intermediate precision (also called inter-assay precision) measure the % RSD for multiple determinations of a single sample, controls and reagents analyzed in several assay runs in the same laboratory.

Procedure:

- Prepare three dilutions of the sample (high/medium/low concentrations in the range).
- Test triplicates of each dilution of the sample inn three different assays.
- Do for day- to –day variations
- Do for lot-to- lot variations of assay materials
- Do for technician – to – technician variation.
- Calculate the average and standard deviation for each point on the curve for each individual test.
- Calculate the RSD for each point on the curve between the assay runs.

Reproducibility refers to the precision between laboratories usually in collaborative studies and not directly relevant to assay validation in a manufacturing facility. Documentation in support of precision studies should include the standard deviation, relative standard deviation, coefficient of variation, and the confidence interval.

C. Specificity

It is the ability of an analytical method to assess unequivocally the analyte of interest in the presence of components that may be expected to be present, such as impurities, degradation products and matrix components. It is not possible to demonstrate that an analytical procedure is specific for a particular analyte. In such case a combination of two or more analytical procedure is recommended to achieve the necessary level of discrimination. Lack of specificity of an individual analytical procedure may be compensated by other supporting analytical procedures or tests.

In case of the assay, demonstration of specificity requires that the procedure is unaffected by the presence of impurities or excipients. In practice, this can be done by spiking the drug substances or product with appropriate levels of impurities or excipients and demonstrating that the assay is unaffected by the presence of these extraneous materials. If the degradation product impurity standards are unavailable, specificity may be demonstrated by comparing the test results of samples containing impurities or degradation products to a second well-characterized procedure e.g., pharmacopoeia method or other validated analytical procedure (independent procedure). These comparisons should include samples stored under relevant stress conditions (e.g. light, heat humidity, acid/base hydrolysis, oxidation.ect.)

D. Limit of Detection

The limit of detection (LOD) is defined as the lowest concentration of an analyte in a sample that can be detected, not quantitated. It is a limit test that specifies whether or not an analyte is above or below a certain value. It is expressed as a concentration at a specified signal-to- noise ratio, usually two –or three-to-one. The ICH has recognized the signal-to-noise ratio convention, but also lists two other options to determine **LOD:** visual non-instrumental methods and a means of calculating the LOD. The method used to determine LOD should be documented and supported, and an appropriate number of samples should be analyzed at the limit to validate the level.

Procedure

- Prepare a standard concentration of the product in the appropriate solution.
- Prepare a blank solution without any sample (zero concentration).
- Perform the assay at least 3 times in duplicate according to SOP.
- Measure the amount present in the sample and blank.
- Calculate the average for the sample and blank.
- Calculate and standard deviation of the blank.
- Calculate the LOD as 3.3xSD/slope of linearity curve.

E. Limit of Quantitation

The limit of quantitation (LOQ) is defined as the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy under the stated operational conditions of the method. That is, as the LOQ concentration level decreases, the precision increases. If better precision is required, a higher concentration must be reported for LOQ.

Procedure:

The calculation method is again based on the standard deviation of the response (SD) and the slope of the calibration curve (S) according t the formula: LOQ = 10(SD/S). Again, the standard deviation of the response can be determined based on the standard deviation of the blank, on the residual standard deviation of the regression line, or the standard deviation of y- intercepts of regression lines.

F. Linearity:

It is ability of an assay to obtain test results, which are directly proportional to the concentration of an analyte in the sample. The determination of linearity will Identify the range of the analytical assay. It can be measured as slope of the regression line and its variance or as the coefficient of determination (R^2) and correlation coefficient(R).

Procedure:

Determining the coefficient of correlation R for dilutions of the sample over the range claimed for assay.

1. Prepare 6 to 8 sample dilutions across the claimed range
2. Test each dilution in triplicate for 3 runs
3. Record expected values, actual values, and % recoveries for each run
4. Analyze each set of dilutions as a linear curve and calculate R for each assay.

Alternative:

Calculate the accuracy and precisions at each dilution. Range is the highest and lowest concentration with satisfactory accuracy and precision. If the validation study for an analytical test is well planed it should be possible to design the protocol to consider many of the parameters in a single series of test, for instance selectivity (specificity) linearity, range, accuracy and precision for a potency test.

G. Range:

Range is the interval between the upper and the lower levels of analyte (inclusive) that have been demonstrated to be determined with precision, accuracy and linearity using the method as written. If the relationship between response and concentration is the linear, the range may be estimated by means of a calibration curve.

The range is normally expressed in the same units as the test results obtained by the method. The ICH guidelines specify a minimum of five concentration levels, along with certain minimum specified ranges. For assay the minimum specified range is from 80-120% of the target concentration. For an impurity test, the minimum range from the reporting level of each impurity, to 120% of the specification. (For toxic or more potent impurities, the range should be commensurate with the controlled level).

H. Ruggedness:

Ruggedness, according to the USP, is the degree of reproducibility of the results obtained under a variety of conditions, expressed as %RSD. The ruggedness of an analytical method is the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of conditions such as different laboratories, different analysts, different instruments, different lot of reagents, different elapsed assay times, different assay temperatures different days, etc.

I. Robustness:

ICH defines robustness as a measure of the method's capability to remain unaffected by small, but deliberate variations in method parameters. Robustness can be partly assured by good system suitability specifications. The evaluation of robustness should be considered during the development phase and depends on the type of procedure under study. It should show the reliability of an analysis with respect to deliberate variations in method parameters. If the results of a method or other measurements are susceptible to variations in method parameters, these parameters should be adequately controlled and a precautionary statement included in the method documentation. One consequence of the evaluation of robustness should be that a series of system suitability parameters (e.g., resolution test) is established to ensure that the validity of the analytical procedure is maintained whenever used.

Examples of typical variation are:

- Stability of analytical solutions
- Extraction time

In the case of liquid chromatography, examples of typical variations are

- Influence of variations of pH in a mobile phase
- Influence of variations in mobile phase composition
- Different columns(different lots and/or suppliers)
- Temperature
- Flow rate.

In the case of gas-chromatography, examples of typical variations are

- Different columns(different lots and/or suppliers)
- Temperature
- Flow rate.

J. System Suitability Test

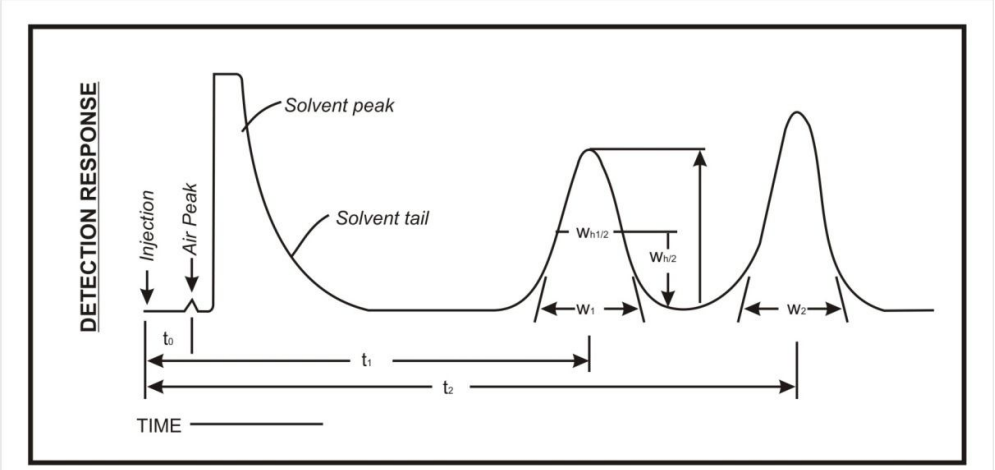
System suitability test is commonly used to verity resolution, column efficiency and repeatability of the chromatographic system to ensure its adequacy for a particular analysis. According to the United States pharmacopoeia (USP) and the International Conference on Harmonization (ICH), SST is an integral part of many analytical procedure.

Primary SST parameters are most important as they indicate system specificity, precision and column stability. Other parameter include capacity factor (K) and signal to noise ratio(S/N) for impurity peaks.

The USP chromatography general chapter states "System suitability test are an integral part of gas and liquid chromatographic methods. They are used to verify that the resolution and reproducibility of the chromatographic system are adequate for the analysis to be done. The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system can be evaluated as such." (USP 36-NF 31, 621 – Chromatography)

INTERPRETASTION OF CHROMATOGRAMS

Figure below represents a typical chromatographic separation of two substances, 1 and 2, where t_1 and t_2 are the respective retention times; and h, h/2, and $W_{h/2}$ are the height, the half – height, and the width at half-height, respectively, for peak1. W1 and W2 are the respective widths of peaks 1 and 2 at the base line. Air peaks are a feature of gas chromatograms and correspond to the solvent front in liquid chromatography.



Chromatography retention times are characteristic of the compounds they represent but are not unique. Coincidence of retention times of a test and a reference substance can be used as a feature in construction of an identity profile but is insufficient on its own to establish identity. Absolute retention times of a given compound vary from one chromatogram to the next.

Relative Retention times:

Relative retention time is calculated by the equation $R_r = t_2/t_1$

t_1 = Retention time of test.

t_2 = Retention time of reference substance, determined under identical experimental conditions on the same column.

Relative Retention:

To calculate the relative retention (r) = $\frac{t_2 - t_M}{t_1 - t_M}$

Where t_M is the retention time of the non-retained marker.

Resolution

The resolution R is a function of column efficiency, N and is specified to ensure that closely eluting compounds are resolved from each other, to establish the

general resolving power of the system, and to ensure that internal standards are resolved from the drug.

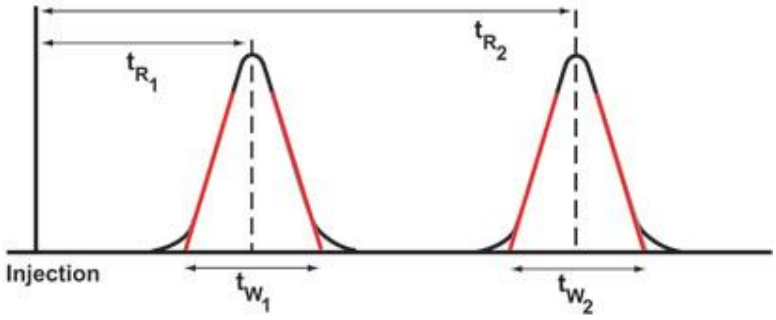


Fig. 2

R is determined by the equation:

$$\mathbf{R}=\frac{2(t_2-t_1)}{W_2+W_1} \quad \text{Or}$$

$$R = \frac{2(t_2-t_2)}{1.70\ (W_{1,\,h/2} + W_{2,\,h/2})}$$

t_2 and t_1 are the retention times of the components.

W_2 and W_1 are the corresponding width at the bases of the peaks obtained by extrapolating the relatively straight sides of the peaks to the base line.

$W_{1h/2}$ and $W_{2h/2}$ are the corresponding peak width at half-height.

Resolution

$$R = \frac{1.18(t_{R2} - t_{R1})}{(W_{h1} + W_{h2})}$$

Where, $t_{R2} > t_{R1}$

$\mathbf{t_{R2}}$ and $\mathbf{t_{R1}}$ = Retention times or distances along the baseline from the point of injection to the perpendiculars dropped from the maxima of two adjacent peak

$\mathbf{W_{h1}}$and $\mathbf{W_{h2}}$ = peak width at half height.

Theoretical Plates

Column efficiency also may be specified as system suitability requirements, especially if there is only one park of interest in the chromatograms. The number of the theoretical plates, N, is a measure of column efficiency. It is calculated by the equation.

$N= 16 [t/w]^2$ or $N= 5.54 [t/w^{1/2}]^2$

t = Retention time of the substance.

w = width of the peak at its base, obtained by extrapolating the relatively straight sides of the peak to the baseline.

$W^{1/2}$ = Peak width at half-height.

Precision:

Precision a measure of either degree of reproducibility or of repeatability is determined by making replicate injections of standard preparation and calculating relative standard deviation. Unless otherwise specified in the individual monograph, data from five replicate injections of the standard preparation are used to calculate the relative standard deviations (SR), if the requirement is 2.0% or less; data from six replicate injections are used if the relative standard deviation requirement is more than 2.0%.

Relative Standard Deviation in percentage.

$$SR\ (\%) = \frac{100}{X}\left[\sqrt{\sum_{i=1}^{i=n} \frac{(Xi-\overline{x)2}}{N-1}}\right]$$

x = Arithmetic mean of the set.

xi = An individual measurement in a set of N measurements.

N= Number of individuals values

Tailing Factor (or) Symmetry factor

Tailing factor, T, a measure of peak symmetry, is unity for perfectly symmetrical peaks and its value increases as tailing factor is pronounced (Fig 1). In some cases values less tan unity may be observed. As peak asymmetry increases, integration and hence precision becomes less reliable.

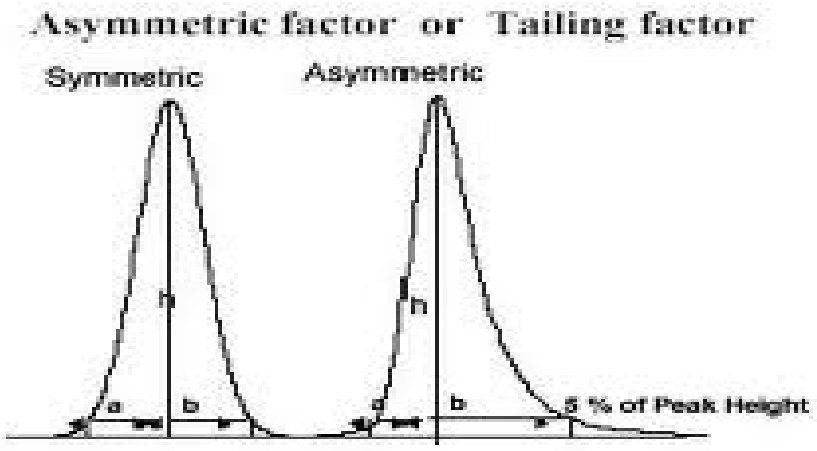


Fig. 3

Tailing factor, T = $\frac{W0.05}{2F}$

W0.05 = Width of peak at 5% height.

F = Distance from the peak maximum to the leading edge of the peak, the distance is being measured at a point 5% of the peak height from baseline.

Capacity Factor (Mass distribution ratio):

Capacity factor k' of a sample component is a measure of the degree which that component is retained by the column relative to an unretained component

Capacity factor is $k' = \frac{(t_{r-t_0})}{t_0}$

t_r – is the elution time of retained component and

t_0 – is the elution time of the unretained sample.

Signal to Noise Ratio:

$$S/N = \frac{2H}{H}$$

Where,

H = Height of the peak corresponding to the component concerned, in the chromatogram obtained with the prescribed reference solution, measured from the maximum of the peak to the extrapolated baseline of the signal observed over a distance equal to 20 times the width at half-height.

h = Range of the background noise in a chromatogram obtained after injection or application of a blank, observed over a distance equal to 20 times the width at half-height of the peak in the chromatogram obtained with the prescribed reference solution and, if possible, situated equally around the place where this peak would be found.

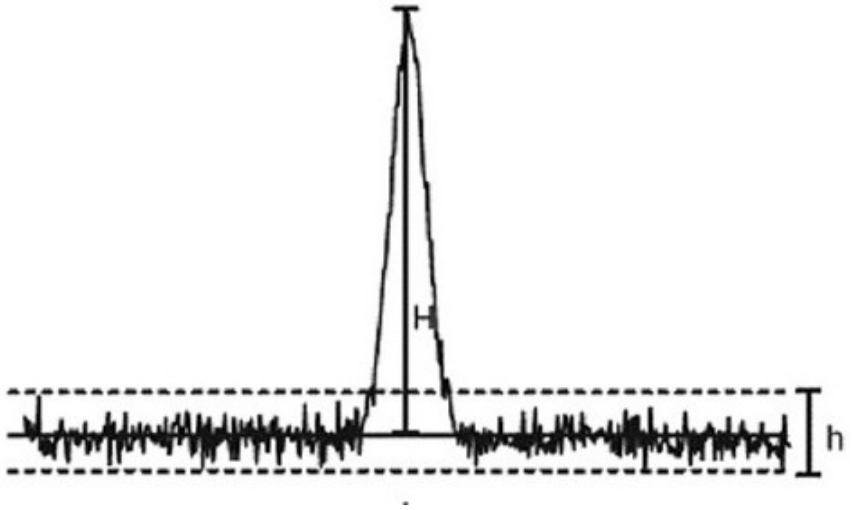


Fig. 4

Peak to Valley ratio

The peak-to-valley ratio (p/v) may be employed as a system suitability requirement in a test for related substances when baseline separation between 2 peaks is not reached

$$P/v = \frac{H_p}{H_v}$$

H_p = Height above the extrapolated baseline of the minor peak,

H_v = Height above the extrapolated baseline at the lowest point of the curve separating the minor and major peaks.(ICH 2007)

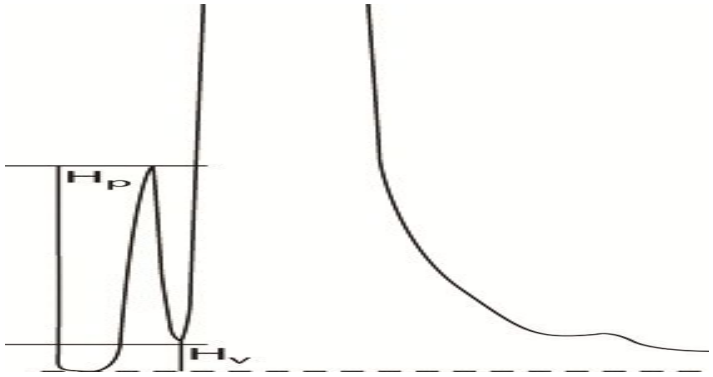


Fig. 5

System Suitability Parameters and Recommendations:

Parameter	Recommendation
Capacity Factor (k')	The peak should be well-resolved from the other peaks and the void volume, generally k'>2.0
Repeatability	RSD, ≤1% for N≥5 is desirable
Relative retention	Not essential as long as the resolution is stated
Resolution	R_s of >2 between the peak of interest and the closed eluting
Tailing Factor (T)	T of ≤2
Theoretical Plates (N)	In general should be > 2000

STATISTICAL PARAMETERS

Linear regression:

Once a linear relationship has been shown to have a high probability by the value of the correlation coefficient 'r', then the best straight line through the data points has to be estimated. This can often be done be done by visual inspection of the calibration graph, but in many cases it is far more sensible to evaluate the best straight line by linear regression (the method of least squares).

The equation of straight line is **y = mx + c**

Where, y the dependent variable is plotted as result of changing x, the independent variable.

To obtain the regression line 'y on x' the slope 'm' of the line and the intercept 'c' on the y axis are given by the following equation.

$$\mathbf{m=\frac{N\sum xy-(\sum x)(\sum y)}{N\sum x2-(\sum x)2}} \quad \text{and} \quad \mathbf{C=\frac{N\sum y\sum x2-(\sum x)(\sum y)}{N\sum x2-(\sum x)2}}$$

Correlation coefficient:

When the changes in one variable are associated or followed by changes in the order it is called correlation. To establish whether there is a liner relationship between two variables x_1 and y_1, use Pearson's correlation coefficient r.

$$r=\frac{n\sum x1y1-\sum x1y1}{\{[n\sum x12-(\sum x1)2][n\sum y12-(\sum y1)2]\}1/2}$$

Where n is the number of data points.

The value of r must lie between +1 and -1, the nearer it is to +1, the greater the probability that a define linear relationship exists between the variables x and y, values close to +1 indicate positive correlation and values close to -1, indicate

negative correlation values of 'r' that tend towards zero indicate that x and y are not linearly related (they made be related in a non-linear fashion).

Standard deviation:

The standard deviation measures the spread f the data about the mean value. It is commonly used in statics as a measure of precision statics as a measure of Precision and is more meaningful than is the average and is expressed mathematically as.

$$S = \sqrt{\sum_{i=1}^{i=n} \frac{(Xi - \bar{x})}{N - 1}}$$

Where,

S is the standard deviation.

If N is large (50 or more) then of course it is immaterial whether the term in the Denomination is N-1 or N

Σ = sum

X = observed values

$\bar{X}$= Mean or arithmetic average = $\Sigma X/N$

X - $\bar{X}$ = deviation of a value from the mean

N = Number of observations

Percentage relative standard deviation

It is also known as coefficient of variation CV. It is defined as the standard deviation (S.D) expressed as the percentage of mean.

$$\text{CV or \% RSD} = \frac{S.D}{\bar{X}} \times 100$$

Where, S.D = Standard deviation,

$\bar{X}$ = Mean or arthimetic average.

The variance is defined as S^2 and is more important in statistics than S itself.

However, the latter is much more commonly used with chemical data.

Standard Error of mean:

Standard error of mean can be defined as the value obtained by division of standard deviation by square root of number of observation. It is mathematically expressed as

$$S.E = \frac{S.D}{\sqrt{n}}$$

Where, n = number of observations.

S.D = Standard Deviation(Riley et.al,2009)

Data elements required for essay validation:

It is not always necessary to evaluate every analytical performance parameter, as different test methods require different validation schemes. The most common categories of assays for which validation data should be required are as follows:

i) Quantitation of major components or active ingredients.

ii) Determination of impurities or degradation compounds.

iii) Determination of performance characteristics

Category – I: Analytical methods for quantitation of major components of bulk drug substances or active ingredients (including preservatives) in finished pharmaceutical products**.**

Category – II: Analytical methods for determination of impurities in bulk drug substances or degradation compounds in finished pharmaceuticals products. These methods include quantitative essays and limits tests.

Category – III: Analytical methods of determination of performance characteristics (E.g. dissolution, drug release).

The type of method and its intended use dictates which parameters are required to be investigated. They are illustrated in the below.

Data elements required for assay validation

Analytical Performance Parameter	**Assay Category-I**	**Assay category**		**Assay Category III**
		Quantitative	**Limit Test**	
Accuracy	Yes	Yes	*	*
Precision	Yes	Yes	No	Yes
Specificity	Yes	Yes	Yes	*
LOD	No	No	Yes	*
LOQ	No	Yes	No	*
Linearity & range	Yes	Yes	No	*
Ruggedness	Yes	Yes	Yes	*

*may be required, depending on the nature of specific test.

Comparison of Analytical Parameters Required for Assay validation

FDA reviewer Guidance	**USP General Chapter <1225>**	**ICH Q2A Guidelines**
Accuracy	Accuracy	Accuracy
Precision Repeatability Analysis	Precision	Precision
Intermediate precision	No	Intermediate precision
Reproducibility	No	No
Specificity / selectivity	Specificity	Specificity
Detection limit	Detection limit	Detection limit
Quantitation limit	Quantitation limit	Quantitation limit
Linearity	Linearity	Linearity
Range	Range	Range
No	Ruggedness	No
Robustness	Robustness	Robustness
System suitability sample solution stability	System suitability	System suitability

LITERATURE REVIEW

First Derivative Spectrophotometric Method for Simultaneous Estimation of Pantoprazole and Cinitapride in Combined Dosage Form.

Patel.P.U. 2014 has reported a new, rapid, precise, accurate and sensitive first derivative spectrophotometric analytical method is proposed for the simultaneous estimation of Pantoprazole and Cinitapride in combined dosage form. The drugs obeyed the Beer's law in the concentration range of 1-26 µg/mL. In the proposed method, absorbance was measured at 302 nm (Zero Crossing Point of Cinitapride) and 249 nm (Zero Crossing Point of Pantoprazole). Accuracy of the method was determined by recovery studies and was found to be 100.35 ± 0.18 and 99.61 ± 0.17 for Pantoprazole and Cinitapride respectively. Therefore the proposed method can be used for routine analysis of both drugs in bulk as well as in pharmaceutical formulations

Development and Validation of dual wavelength method for simultaneous estimation of Omeprazole and Cinitapride in combined capsule dosage form.

Jagani.N.M 2012 has reported simple, sensitive, rapid, accurate and precise dual wavelength spectrophotometric method described for the simultaneous estimation of Omeprazole (OMP) and Cinitapride Hydrogen Tartrate (CNT) in combined dosage form. The method was based on determination of OMP at the absorbance difference between 275 nm and 255 nm and CNT at the absorbance difference between 288 nm and 312.9 nm. The linearity was obtained in the concentration range of 10-30 µg/mL and 1.5-4.5 µg/mL for OMP and CNT respectively. The proposed method was found to be simple and sensitive for the

routine quality control application of OMP and CNT in capsule dosage form. The results of analysis have been validated statistically and by recovery studies.

Determination of Mosapride and Pantoprazole in a fixed-dose combination by UV Spectrophotometric methods and RP-HPLC

Birajdar. A.S 2011 has reported An accurate and reproducible UV-spectrophotometric methods and liquid chromatographic assay method were developed and validated for the determination of mosapride and Pantoprazole in capsule formulation. Two wavelengths were selected for each UV method, first simultaneous equation 274 nm, 288.2 nm and second Q value analysis method 274 nm, 302 nm was the isobestic point for both the drugs. The 30 mM ammonium sulphate buffer: acetonitrile (50:50, v/v) was used for RP-HPLC to determine the contents of mosapride and Pantoprazole in combination-capsule dosage form. Linearity was evaluated over the concentration range of 5-50.0 µg/mL by UV and 0.5 to 5.0 µg/mL by HPLC method respectively, for mosapride and Pantoprazole (the value of R2 0.999 found were by both the methods for mosapride and Pantoprazole).

Simultaneous Spectrophotometric Estimation of Cinitapride Hydrogen Tartarate and Omeprazole in Capsule Dosage Form

Satish.P 2013 has reported two simple, sensitive, rapid, accurate, precise and economical spectrophotometric methods were developed for the simultaneous estimation of Cinitapride hydrogen tartarate and omeprazole in two components solid dosage form. First method is based on the simultaneous equation and second method is based on Q-analysis (absorbance ratio method). Cinitapride hydrogen tartarate has absorbance maxima at 265 nm and omeprazole has absorbance maxima at 301 nm in methanol. The linearity was obtained in the concentration range of 1-20 µg/mL for both Cinitapride hydrogen tartarate and omeprazole. In the first method, the

concentrations of the drugs were determined by using simultaneous equations and in second method, the concentration of the drugs were determined by using ratio of absorbance at isoabsorptive point and at the λ-max of one of the drug. The results of analysis have been validated statistically and by recovery studies.

Development and Validation of RP-HPLC method for simultaneous estimation of Naproxen and Pantoprazole in pharmaceutical dosage form.

Kumar.R 2011 has reported a simple, sensitive, and precise high performance liquid chromatographic method for the analysis of naproxen and Pantoprazole has been developed, validated and used for the determination of compounds in commercial pharmaceutical products. The compounds were well separated on a Hypersil BDS C-18 reversed-phase column by use of a mobile phase consisting of 0.1 M sodium acetate (pH 8.2), acetonitrile and methanol (70:20:10 v/v) at a flow rate of 1.0 mL min-1 with detection wavelength at 285 nm. The linearity ranges were 5-70 µg mL-1 for naproxen, and 5-40 µgmL-1 for Pantoprazole. The recovery amount was more than 99 %. The high recovery and low relative standard deviation confirms the suitability of the method for determination of naproxen and Pantoprazole in pharmaceutical dosage forms.

Development and Validation of RP-HPLC for the Pantoprazole Sodium Sesquihydrate in Pharmaceutical dosage forms and Human Plasma.

Prasanna Reddy B 2012 has reported A simple, sensitive and precise HPLC method for the analysis of Pantoprazole sodium and lansoprazole has been developed, validated and used for the determination of compounds in commercial pharmaceutical products. The compounds were well separated an isocratically on a C18 column [Use Inertsil C18, 5m , 150 mm x 4.6 mm] utilizing a mobile phase consisting of acetonitrile: phosphate buffer (60:40, v/v, pH 7.0) at a flow rate of 1.0 mL/min with

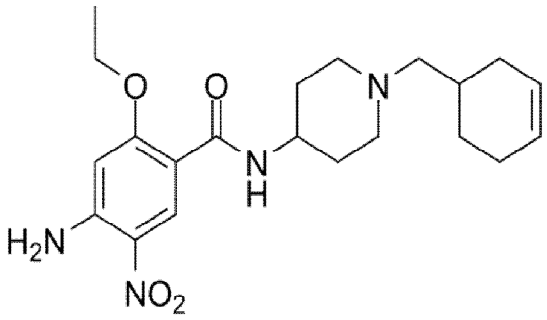
UV detection at 230 nm. The retention time of Pantoprazole sodium and lansoprazole was found to be 2.017 min and 2.538. The study showed that reversed-phase liquid chromatography is sensitive and selective for the determination of Pantoprazole sodium and lansoprazole using single mobile phase.

Spectrophotometric methods for simultaneous estimation of pantoprazole and itopride hydrochloride in capsules

Gupta K.R 2012 has reported, three simple, accurate and economical methods for simultaneous estimation of Pantoprazole and itopride hydrochloride in two component solid dosage forms have been developed. The proposed methods employ the application of simultaneous equation method, absorbance ratio method and multicomponent mode of analysis method. In distilled water Pantoprazole shows λmax at a wavelength of 289.0 nm while itopride hydrochloride shows λmax at a wavelength of 258.0 nm also the drugs show an isoabsorptive point at a wavelength of 270.0 nm. For multicomponent method, sampling wavelengths 289.0 nm, 270.0 nm and 239.5 nm were selected. All these methods showed linearity in the range from 4-20 µg/mL and 15-75 µg/mL for Pantoprazole and itopride hydrochloride respectively. The results of analysis have been validated statistically and by recovery studies.

CINITAPRIDE

Structure :



Molecular Formula : $C_{21}H_{30}N_4O_4$

IUPAC name : 4-amino-N-[1-(cyclohex-3-en-1-ylmethyl) piperidin- 4 yl]-2-ethoxy-5-nitrobenzamide

Molecular weight : 402.4873

Description : Yellow crystalline powder.

Solubility : Sparingly soluble in water & in glacial acetic acid (1 in 100).

pKa : 16.32

Category : Anti emetic.

Pharamacokinetic data:

- Absorption **:** The absorption of Cinitapride (12mg) following oral administration was rapid, with peak levels being achieved 2 h after dosing; absorption following intramuscular administration (4mg) was even more rapid, with peak levels (50% more that oral levels) being achieved 1 h after dosing.

- Metabolism **:** Metabolism is reported through cytochrome P450 CYP3A4 and CYP2C8 isozymes. Renal Excretion accounts for minor
- Elimination **:** The elimination profile was similar in oral and intramuscular administration. Renal route of elimination is minor. Urinary 24-h excretion of Cinitapride and its two major metabolites (principally the de-alkylated product) was no greater than 7% of the administered dose.
- Half-life **:** 3-5 h during the first 8 h and a residual half-life greater than 15 h thereafter.

Mechanism of action:

Cinitapride is a substituted benzamide used for its prokinetic properties with 5-HT receptor antagonist and agonist activity. (S.C Sweetman 2005)

Toxicity:

The symptoms of overdose include drowsiness, confusion and extra pyramidal effects.

Uses:

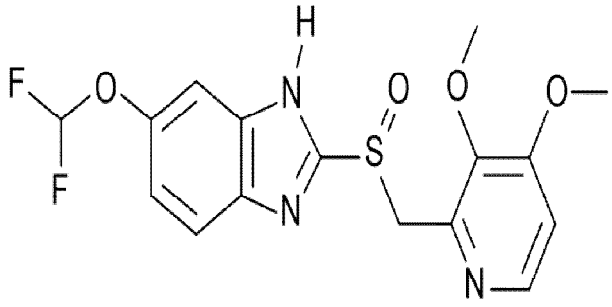
- For the treatment of gastrointestinal disorders associated with motility disturbances such as GERD, non-ulcer dyspepsia and delayed gastric emptying.

Storage:

Store between 15°C-25°C and protect from Moisture

PANTOPRAZOLE

Structure :



Molecular Formula : $C_{16}H_{15}F_2N_3O_4S$

IUPAC Name : 6-(difluoromethoxy)-2-{[(3,4-dimethoxypyridin-2-yl) methane] sulfinyl}-1H-1, 3-benzodiazole

Molecular weight : 383.37

Description : White to off-white powder

Solubility : Freely soluble in water, in methanol, and in dehydrated Alcohol; practically insoluble in hexane and in Dichloromethane.

pKa : 15.76

Category : Proton pump inhibitor

Mechanism of action :

Pantoprazole is a proton pump inhibitor (PPI) that suppresses the final step in gastric acid production by forming a covalent bond to two sites of the (H^+,K^+)-ATPase enzyme system at the secretory surface of the gastric parietal cell. This effect is dose- related and leads to inhibition of both basal and stimulated gastric acid secretion irrespective of the stimulus. (C.F. Lacy 2005)

Pharamacokinetic data:

Absorption:	Pantoprazole is well absorbed. It undergoes little first-pass metabolism resulting in an absolute bioavailability of approximately 77%.
Volume of Distribution:	11.0 to 23.6 L+
Protein binding:	98% primarily to albumin
Metabolism:	Pantoprazole is extensively metabolized in the liver through the cytochrome P450 (CYP) system. The main metabolic pathway is demethylation, by CYP2C19, with subsequent sulfation; other metabolic pathways include oxidation by CYP3A4. There is no evidence that any of the Pantoprazole metabolites have significant pharmacologic activity.
Elimination:	After administration of a single intravenous dose of 14C-labeled Pantoprazole to healthy, normal metabolizer subjects, approximately 71% of the dose was excreted in the urine with 18% excreted in the feces through biliary excretion
Half-life:	1 hour

Uses:

Short-term (up to 16 weeks) treatment of erosive esophagitis

Storage:

Store in well-closed light-resistant containers at 15-30 ^{0}C

AIM AND PLAN OF WORK

The aim of the work is to develop a new method for the analysis of Cinitapride and Pantoprazole by RP–HPLC in capsule dosage form and to validate it as per ICH guidelines**.**

The drugs analysis plays an important role in the development of drugs, their manufacture and therapeutic use. Pharmaceutical industries rely upon quantities chemical analysis to ensure that the raw material used and the final product obtained meets the required specification. The number of drugs and drug formulations introduced into the market has been increasing at an alarming rate. These drugs or formulations may be either new entities in the market or partial structural modification of the existing drugs or novel dosage forms or multi component dosage forms.

The multi component dosage forms proved to be effective due to combined mode of action on the body. The complicity of including the presence of multiple drug entities posses considerable challenge to the analytical chemist during the development of assay procedure. The estimation of individual drugs in these multi component dosage forms becomes difficult due to some extraction or isolation procedures.

For the present study Cinitapride and Pantoprazole was selected. The extensive literature survey carried out revealed that there is no method reported for the simultaneous estimation of these drugs, some methods for estimation of combined drugs by HPLC and spectrophotometer are available. Hence present study aim to developing a specific, precise, accuracy, linear, simple, rapid, and validated and cost effective RP-HPLC method for the simultaneous estimation of these drugs in combined dosage forms.

The core of project for RP-HPLC method was designed as follows:

1. Selection of suitable wavelength,

2. Selection of stationary phase and mobile phase,

3. Selection of initial separation conditions,

4. Optimization of chromatographic conditions,

5. Estimation of Cinitapride and Pantoprazole

6. Method validation.

MATERIALS AND METHODS

MATERIALS USED FOR METHOD DEVELOPMENT AND VALIDATION

INSTRUMENT USED:

HPLC	:	Agilent 1260 series
Software	:	Open lab
pH Meter	:	Range from 0 -14.Deep vision
Analytical balance	:	Accurate to 0.001g- Mettler
Solvent filtration Unit	:	Milli pore
Syringe filters	:	Nylon 0.22µ filter, PVDF 0.45 µ filters
Centrifuge	:	Remi
Sonicator	:	1.5LH ultrasonic bath.

APPARATUS USED:

Graduated cylinder	:	50mL, 100mL, and 1000mL – Borosil.
Volumetric flask	:	200mL, 100mL, 50mL –Borosil.
Volumetric pipettes	:	1mL, 10mL, 5mL, 4mL, 20mL –Borosil.
Graduate pipettes	:	5mL, 10mL, 1mL, 2mL –Borosil

Specification	
Column	C_{18} (Zorbax ODS Column 250mmX 4.6mm), 5µ
Detector	DAD
Temperature	Ambient
Wavelength	281 nm

Chemicals Used:

Methanol	: Merck HPLC grade
Acetonitrile	: Merck HPLC grade
Sodium hydroxide	: Merck AR grade
Water	: Milli Q
Potassium dihydrogen phosphate	: Merck AR grade
Ortho phosphoric acid	: Merck AR grade

Standard Used

- CinitaprideWorking Standard of Known potency
- PantoprazoleWorking Standard of Known Potency

Source of drugs:

- Samples were collected from Avon organics

METHOD DEVELOPMENT

Selection of Mobile Phase

The pure drugs Cinitapride and Pantoprazole were injected in combination in the ratio of their contents in the capsule formulation to the chromatographic system, and run in different mobile phase compositions. Different mobile phases containing different compositions of methanol: water, acetonitrile: water, and acetonitrile: Phosphate buffer were tried for the selection of optimum conditions for the simultaneous determination of Cinitapride and Pantoprazole. It was found that optimal separation of the two components was achieved with acetonitrile and phosphate buffer (KH_2PO_4 buffer), compared to other mobile phases. Different ratios of the selected mobile phase were tried with varying flow rates and pH. Finally, the mobile phase composition selected for the chromatographic separation of Cinitapride and Pantoprazole was acetonitrile and phosphate buffer of pH 6.8 in the ration of 50:50 v/v.

Preparation of Mobile Phase

Phosphate buffer pH 6.8: 50mL of 0.2M KH_2PO_4 and 22.4mL of 0.2M NaOH were dissolved in 100mL of distilled water.

Mobile phase was prepared by mixing 500 mL of acetonitrile with 500 mL of phosphate buffer and its pH adjusted to 6.8. The mobile phase was sonicated for 15 min and filtered through a 0.45 µm membrane filter paper.

Preparation of Standard Stock Solutions

25 mg each of standard Cinitapride and Pantoprazole were weighed accurately and transferred in to two separate 25mL flasks, and dissolved in 10mL of solvent, the volume was made up to the mark with solvent to obtain a solution of concentration of 1000 µg/mL of each Cinitapride and Pantoprazole (standard stock solutions A1 and

A2 respectively). From the above stock solution A1 and A2 respectively 1.5mL and 20 mL aliquots were pipetted in to a 50mL volumetric flask and dissolved in 25mL of the solvent and made up to the mark with the solvent to obtain a final concentration of 400 and 30µg/mL of Pantoprazole and Cinitapride respectively (working stock solution). The stock solution were filtered through a 0.45µm membrane filter and sonicated for 15min in an ultrasonic bath sonicator.

Preparation of Standard Solution:

Transfer 5 mL of standard stock solution into a 20 mL volumetric flask, and dilute with diluent.

Selection of Analytical Wavelength

UV Spectrophotometric determination of Cinitapride and Pantoprazole of concentration 20µg/mL individually, in overlay mode shows that both the drugs absorb appreciably at 281nm, hence 281nm was selected as the detection wavelength (Fig.: 6).

Selection of Analytical Concentration Range and Preparation of Calibration Curves for Cinitapride and Pantoprazole

Appropriate aliquots of 0.5, 1, 1.5, 2, 2.5, 3 and 3.5mL were pipetted out from the standard stock solution in to a series of 10mL volumetric flasks. The volume was made up to the mark with the solvent resulting in the concentrations ranging from 1.5 to 10.5 µg/mL Cinitapride and 20-140 µg/mL Pantoprazole. These seven level concentrations were prepared in triplicate and 20µl of these solutions were injected to chromatographic system. Peak areas respective to each concentration were recorded at 281nm and a calibration graph of concentration vs. peak area was established.

Both the drugs follow the beer's lamberts law in the concentration range of 1.5-10.5 µg/mL Cinitapride and 20-140 µg/mL Pantoprazole. The regression analysis

was carried. The linearity of the peak response and adherence of the system to beers lamberts law was validated by high value of correlation coefficient and a % RSD of less than 2.0.

Analysis of Capsule Formulation:

Preparation of Sample Stock Solution:

The contents of twenty marketed Cintodac capsules were weighed accurately and their average weight was determined. A mass equivalent to 40 mg Pantoprazole and 3mg Cinitapride from the contents of the capsule were taken in a 100mL volumetric flask and dissolved in 50mL of the solvent. The solution was kept for sonication 15min. The solution was made up to the mark with the solvent and filtered through a 0.45 µ membrane filter paper sample stock solution 'A'.

Preparation of Sample Solution:

Transfer 5.0 mL aliquot of the working sample stock solution A was diluted to 20 mL to obtain a concentration of 7.5 and 100µg/mL of Cinitapride and Pantoprazole respectively.

Six replicates solutions of this concentration were prepared. A 20µL volume of these solutions were injected to the chromatographic system and their respective chromatograms were recorded at 281nm. From the peak areas the amount of drug present in each sample was determined.

The objective of this experiment was to optimize the assay method for estimation of Cinitapride and Pantoprazole based on the literature survey and the trails made. The trials mentioned below describes how the optimization was done.

Trial-1:

Mobile Phase	:	Methanol and Water were mixed in the ratio of50:50 and Sonicated to degas.
Diluent	:	Mobile phase

Chromatographic condition:

Column	:	C_{18}Zorbax ODS (250 x 4.6mm,5µ)
Column Temperature	:	Ambient
Flow rate	:	1.0ml/min
Injection volume	:	20µl
Detector wave length	:	281nm
Run time	:	10 min
Observation	:	Peaks are not eluted properly (Fig.: 7)

Trial 2:

Mobile Phase	:	ACN and Water were mixed in the ratio of50:50 and Sonicated to degas.
Diluent	:	Mobile phase

Chromatographic condition :

Column	:	C_{18}Zorbax ODS (250 x 4.6mm, 5µ)
Column Temperature	:	Ambient
Flow rate	:	1.0ml/min
Injection volume	:	20µl
Detector wave length	:	281nm
Run time	:	10 min
Observation:		Merging of peaks was observed(Fig.: 8)

Trial 3:

Mobile Phase	:	Acetonitrile and Phosphate buffer (pH 6.8) were mixed in the ratio of 50:50 and Sonicated to degas.
Diluent	:	Mobile phase
Chromatographic condition	**:**	
Column	:	C_{18}Zorbax ODS (250 x 4.6mm, 5µ)
Column Temperature	:	Ambient
Flow rate	:	1.0ml/min
Injection volume	:	20µl
Detector wave length	:	281nm
Run time	:	10 min
Observation:		Peaks were well resolved with good resolution,tailing and theoretical plate count. (Fig.: 9)

OPTIMIZED HPLC METHOD

After several trials with the different combinations and ratios of solvents, chromatographic parameters of trial-3 were optimized.

Preparation of Mobile phase:

Acetonitrile and Phosphate buffer (pH 6.8) were mixed in the ratio of 50:50 and sonicated to degas.

Chromatographic condition	**:**	
Column	:	Zorbax ODS (250 x 4.6mm, 5µ)
Column Temperature	:	Ambient

Flow rate	:	1.0ml/min
Injection volume	:	20µl
Detector wave length	:	281nm
Run time	:	10 min

Conclusion:

Good separation and resolution between Cinitapride and Pantoprazole peaks was observed.

Procedure:

Equilibrate the column with mobile phase for not less than 30 minutes at a flow rate of 1.0ml/min. Separately inject 20µl of diluents as blank, standard solution (six times) and sample solution into the chromatographic system. Record the chromatograms and measure the peak responses.

System suitability: The column efficiency as determined for the Cinitapride and Pantoprazole peak from standard solution is NLT 2000 theoretical plates**.**

Tailing factor for Cinitapride and Pantoprazolepeak obtained from standard chromatogram should be NMT 2.0 The % RSD for the Cinitapride and Pantoprazole peaks for 6 replicate injections of standard solution should be NMT 2.0

The retention time of Cinitapride peak is about 4.5 minutes and Pantoprazole peak is about 5.4 minutes

Calculations:

Quantity of Cinitapride and Pantoprazole present in the capsule as % of labeled amount:

For CINITAPRIDE:

$$= \frac{A_T \times W_S \times 1.5 \times 5x100 \, x20 \times P \times AW \times 100}{A_S \times 25 \times 50 \times 20 \, xW_T \times 5x100 \times LC}$$

Where,

A_T: Area of Cinitapride peak in sample solution

A_S: Average area of Cinitapride peak obtained from six replicate injectionsof standard solution.

Ws: Weight of Cinitapride working standard taken, in mg

W_T: Weight of sample taken, in mg

P:Purity of Cinitapride working standard used

LC:Label claim of Cinitapride, in mg per capsule

AW: Average fill weight of capsule in mg.

For PANTOPRAZOLE:

$$= \frac{A_T \times W_s \times 20 \times 5 \, x100 \, x20x \, P \times AW \times 100}{A_S \times 25 \times 50 \times 20 \, xW_T \times 5x \, 100 \times LC}$$

Where,

AT: Area of Pantoprazolepeak in sample solution

As: Average area of Pantoprazole peak obtained from six replicate injectionsof standard solution.

Ws: Weight of Pantoprazoleworking standard taken, in mg

W_T: Weight of sample taken, in mg

P: Purity of Pantoprazole working standard used

LC :Label claimPantoprazole, in mg per capsule

AW: Average fill weight of capsule in mg.

METHOD VALIDATION

Preparation of Standard Stock Solution:

25 mg each of standard Cinitapride and Pantoprazole were weighed accurately and transferred in to two separate 25mL flasks, and dissolved in 10mL of solvent, the volume was made up to the mark with solvent to obtain a solution of concentration of 1000 µg/mL of each Cinitapride and Pantoprazole (standard stock solutions A1 and A2 respectively). From the above stock solution A1 and A2 respectively 1.5mL and 20 mL aliquots were pipetted in to a 50mL volumetric flasks and dissolved in 25mL of the solvent and made up to the mark with the solvent to obtain a final concentration of 400 and 30µg/mL of Pantoprazole and Cinitapride respectively (working stock solution). The stock solutions were filtered through a 0.45µm membrane filter and sonicated for 15min in an ultrasonic bath sonicator.

Preparation of Standard Solution:

Transfer 5mL aliquot of the working standardstock solution A was diluted to 20 mL to obtain a concentration of 7.5 and 100µg/mL of Cinitapride and Pantoprazole respectively.

Preparation of Sample Stock Solution:

The contents of twenty marketed Cintodac capsules were weighed accurately and their average weight was determined. A mass equivalent to 40 mg Pantoprazole and 3mg Cinitapride from the contents of the capsule were taken in a 100mL volumetric flask and dissolved in 50mL of the solvent. The solution was kept for sonication 15min. The solution was made up to the mark with the solvent and filtered through a 0.45 µ membrane filter paper sample stock solution 'A'.

Preparation of Sample Solution:

Transfer 5.0 mL aliquot of the working sample stock solution A was diluted to 20 mL to obtain a concentration of 7.5 and 100µg/mL of Cinitapride and Pantoprazole respectively.

SYSTEM SUITABILITY:

The chromatographic system was equilibrated using the initial mobile phase composition, followed by 6 injections of the standard solution of concentration 7.5 µg/mL Cinitapride and100 µg/mL Pantoprazole. The system suitability parameters including Theoretical plates, resolution, tailing factor and asymmetric factor were determined. The results were statistically analysed for the determination of standard deviation and %RSD.

Table: 1 Data of system suitability chromatograms

S.No	Retention time		Peak area	
	CIN	**PAN**	**CIN**	**PAN**
1	4.547	5.397	7968111	92478206
2	4.537	5.397	8085965	93227923
3	4.540	5.400	7901974	92562832
4	4.537	5.403	7938349	91486225
5	4.530	5.407	7871160	91603127
6	4.527	5.400	8062795	93050876
Mean	4.535	5.400	7971392	92401532
SD	0.005	0.006	86544.1	722731.5
% RSD	0.12	0.12	1.09	0.78
No. of Theoretical Plates			13127	14273
Resolution			2.79	
Tailing factor			1.1	1.3

Table: 2 System suitability parameters

Parameters	CIN	PAN
Retention Time (min)	4.535	5.400
Tailing	1.1	1.3
Resolution	-	2.79
Theoretical Plates	13127	14273
%RSD	1.09	0.78

Observation:

From the system suitability studies it was observed that all the parameters are within limit, hence it is concluded that the Instrument, Reagents and Column are suitable to perform Assay.

Acceptance criteria

The % RSD of Cinitaprideand Pantoprazole peak areas should be NMT 2%.

The number of theoretical plates (N) for the Cinitaprideand Pantoprazole peaks is NLT 2000.

The Tailing factor (T) for the Cinitaprideand Pantoprazole peaks is NMT 2.0.

LINEARITY

Aliquots of 0.5, 1, 1.5, 2, 2.5, 3, 3.5 mL of working stock solution was serially diluted to 10mL in separate volumetric flasks to obtain a solution of concentrations in the range 1.5-10.5µg/mL of Cinitapride and 20-140µg/mL of Pantoprazole.

The prepared solutions with increasing concentrations of Cinitapride and Pantoprazole reference standards were analysed chromatographically. For every concentration in µg/mL was measured the peak response value at 281nm. The experimental results were subjected to linear regression analysis.

The drugs Cinitapride and Pantoprazole follow the beer's lamberts law in the concentration range of 1.5-10.5 µg/mL and 20-140 µg/mL respectively. Linearity determinations were carried in triplicate (n=3). Regression equation was established and the correlation coefficient was determined.

Calibration Curve

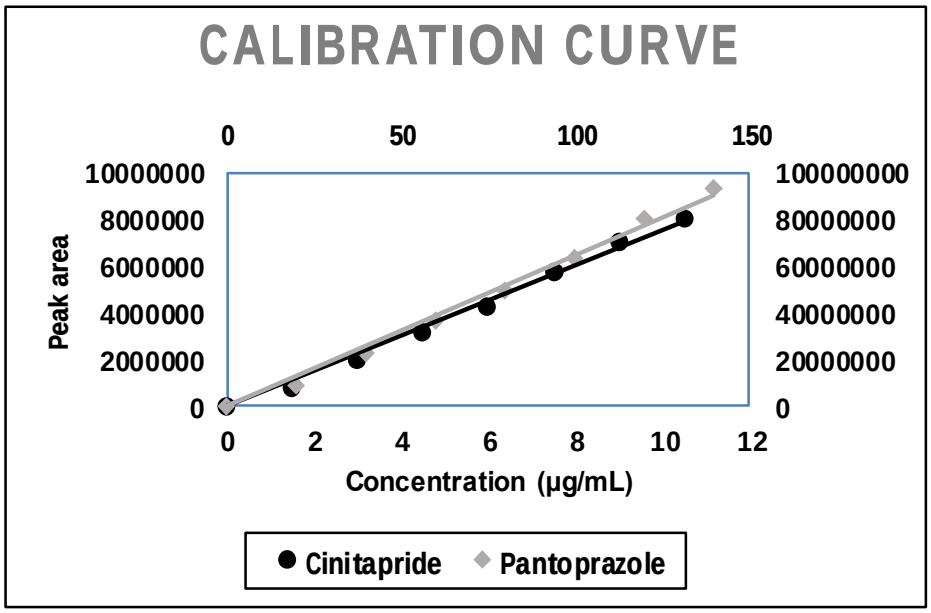


Fig.: 10

Table 3 HPLC Linearity Data of Cinitapride and Pantoprazole

Cinitapride			
Concentration µg/mL	**Peak area n=1**	**Peak area n=2**	**Peak area n=3**
Level 1-1.5	1271341	1294718	1095714
Level 2-3.0	2302253	2376086	2176603
Level 3-4.5	3518631	3650508	3155130
Level 4-6.0	4729404	4848928	4248871
Level 5-7.5	5923659	5814888	5328019
Level 6-9.0	7265947	7157380	6251377
Level 7- 10.5	8155958	8168715	7368677
Correlation coefficient	0.9995	0.9996	0.9999
Slope	786823.2	777850.3	697674.94
y- intercept	15077.17	80188.67	40255.41
Pantoprazole			
Level 1-20	10005542	10703227	11703227
Level 2-40	25379145	23830602	24832691
Level 3-60	38938675	36537859	37765210
Level 4-80	52442612	49024228	49417502
Level 5-100	65873861	63834941	64910451
Level 6-120	81936017	77674361	78676174
Level 7-140	93249699	91640930	93641899
Correlation coefficient	0.9993	0.9993	0.9992
slope	682969.3	660024.74	668002.1
y intercept	-1829654.167	-2045963.41	-1641755.75

Table 4 Statistical Linearity Validation Data of Cinitapride and Pantoprazole

Parameter	Cinitapride	Pantoprazole
Linearity (µg/mL)	1.5-10.5	20-140
Correlation coefficient	0.9997	0.9993
Slope	754116.2	670332
y intercept	45173.75	-1839124
Limit of detection (µg/mL)	0.144	0.996
Limit of quantitation (µg/mL)	0.431	2.987

ACCURACY:

Procedure for the Determination of Accuracy

Recovery studies were performed by applying standard addition method. To a known amount of the pre-analysed drug sample an 80%, 100%, and 120% of standard drug substance was added and suitably diluted. The peak areas of the resultant solutions were measured at 281nm. The amount recovered at each recovery level was determined by substituting the peak response values in the regression equation.

In 80% recovery level concentration the amount of standard added was 2.4mg Cinitapride and 32mg Pantoprazole (80% addition). In 100% recovery level concentration the amount of standard added was 3mg Cinitapride and 40mg Pantoprazole (100% addition). In 120% recovery level concentration the amount of standard added is 3.6mg Cinitapride and 48mg Pantoprazole (120% addition). To each of the above three recovery levels a sample concentration equivalent to 0.6mg of Cinitapride and 8mg of Pantoprazole of the capsule dosage form was added.

The contents corresponding to the three recovery levels were transferred in to three separate 100mL volumetric flasks and dissolved in a small quantity of methanol and sonicated in an ultrasonic bath sonicator for 10min. finally the volume was made up to the volume with methanol. This solution was filtered through Whatman filter paper No. 41.

The solution corresponding to the three recovery levels were prepared in triplicates. A 20µL volume of these solutions were injected to the chromatographic system and the respective chromatograms were recorded at 281nm. The %recovery at each level was calculated by substituting the peak area values in the regression equation and the results were statistically validated.

Table 5 Data of Assay chromatograms

S.No	Retention time		Peak area	
	CIN	PAN	CIN	PAN
1	4.520	5.397	7925458	92781379
2	4.526	5.391	8008349	93005443
3	4.538	5.400	7891102	92620009
4	4.540	5.408	8002877	91753925
5	4.529	5.411	8024688	91980341
6	4.533	5.397	7973557	92706273

Table 6Assay data of Cintodac Marketed Formulations

S.No.	Conc. present (µg/mL)		Conc. obtained (µg/mL)		% recovery (%w/w)	
	CIN	**PAN**	**CIN**	**PAN**	**CIN**	**PAN**
1	7.5	100	7.472	99.77	99.63	99.77
2	7.5	100	7.550	100.01	100.67	100.01
3	7.5	100	7.440	99.60	99.20	99.60
4	7.5	100	7.545	98.67	100.60	98.67
5	7.5	100	7.565	98.91	100.87	98.91
6	7.5	100	7.517	99.69	100.23	99.69
Statistical validation data						
Drug		**Mean**		**Standard deviation**		**%RSD**
Cinitapride		7.515		0.05		0.66
Pantoprazole		99.44		0.53		0.53

Table 7Accuracy Data of CIN and PAN

Recovery level	Amount of standard		Amount of test added		Total amount recovered		% Recovery	
	CIN	**PAN**	**CIN**	**PAN**	**CIN**	**PAN**	**CIN**	**PAN**
80%	2.4	32	0.6	8	3.04	39.59	101.5	98.9
	2.4	32	0.6	8	2.99	39.41	99.8	98.5
	2.4	32	0.6	8	3.01	40.09	100.5	100.2
100%	3.0	40	0.6	8	3.61	48.21	100.5	100.4
	3.0	40	0.6	8	3.56	48.11	99.1	100.2
	3.0	40	0.6	8	3.64	48.68	101.1	101.4
120%	3.6	48	0.6	8	4.28	56.11	101.9	100.2
	3.6	48	0.6	8	4.20	56.76	100.2	101.4
	3.6	48	0.6	8	4.24	56.77	101.2	101.4

Table 8 Statistical Validation Data of Accuracy

Level of recovery	Mean		Standard deviation		% RSD	
	CIN	**PAN**	**CIN**	**PAN**	**CIN**	**PAN**
80 %	3.018	39.698	0.0258	0.3504	0.856	0.882
100 %	3.608	48.339	0.0372	0.3070	1.032	0.635
120%	4.245	56.551	0.0368	0.3787	0.867	0.669

PRESICION

Procedure for the Determination of Precision:

The precision of the analytical method was determined a minimum of 6 determinations at the 100% test concentrations. An amount equivalent to 3mg Cinitapride and 40mg of Pantoprazole was weighed accurately and transferred to a 100mL volumetric flasks and dissolved in a small quantity of solvent and the content was kept in a sonicator for 10min. finally the volume was made up to the mark with the solvent. The solution was filtered through 0.45µ Nylon filter. The above sample solution was suitably diluted with the solvent to obtain a solution of concentration 7.5µg/mL Cinitapride and 100µg/mL Pantoprazole.

Intra-day Precision:

In intraday precision six replicate sample matrices containing 7.5µg/mL Cinitapride and 100µg/mL Pantoprazole were chromatographically analysed at different time intervals on the same day. The variation of the results within the same day was analysed and statistically validated.

Table 9 Intraday Precision Areas of CIN and PAN

S.No	Retention time		Peak area	
	CIN	PAN	CIN	PAN
1	4.523	5.387	8002877	92081040
2	4.529	5.411	7978349	92005111
3	4.514	5.401	7991125	91127487
4	4.522	5.384	8022087	91237418
5	4.529	5.408	7924688	91304394
6	4.505	5.391	7903049	93004714

Table 10 Intraday Precision Data of CIN and PAN

S. No	Conc. present (µg/mL)		Result in mg/mL		Result in %	
	CIN	**PAN**	**CIN**	**PAN**	**CIN**	**PAN**
1	7.5	100	3.005	40.03	100.59	99.71
2	7.5	100	2.996	39.99	99.77	100.32
3	7.5	100	3.001	39.61	101.50	101.27
4	7.5	100	3.012	39.66	101.15	100.97
5	7.5	100	2.976	39.69	99.07	101.72
6	7.5	100	2.968	40.43	100.28	100.16

Table 11 Statistical Validation Data of Intraday Precision

Drug component	Mean	Standard deviation	%RSD
Cinitapride	99.77 %	0.58	0.58
Pantoprazole	99.76 %	0.78	0.78

Inter-day Precision:

In inter-day precision six replicate sample matrices containing 7.5µg/mL Cinitapride and 100µg/mL Pantoprazole were chromatographically analysed on three consecutive (1^{st}, 2^{nd} and 3^{rd}) days. The variation of the results within the same day was analysed and statistically validated.

Table: 12 Data of Interday precision chromatograms

S.No	Retention time		Peak area	
	CIN	PAN	CIN	PAN
1	4.520	5.397	7925458	92781379
2	4.526	5.391	8008349	93005443
3	4.538	5.400	7891102	92620009
4	4.540	5.408	8002877	91753925
5	4.529	5.411	8024688	91980341
6	4.533	5.397	7973557	92706273

Table 13 Inter day Precision Data CIN & PAN

Sl. No	Conc. present (µg/mL)		Result in mg/mL		Result in %	
	CIN	PAN	CIN	PAN	CIN	PAN
1	7.5	100	2.989	39.91	99.63	99.77
2	7.5	100	3.020	40.00	100.67	100.01
3	7.5	100	2.976	39.84	99.20	99.60
4	7.5	100	3.018	39.47	100.60	98.67
5	7.5	100	3.026	39.56	100.87	98.91
6	7.5	100	3.007	39.88	100.23	99.69

Table 14 Statistical Validation Data of Inter day Precision

Drug component	Mean	Standard deviation	%RSD
Cinitapride	100.20 %	0.65	0.65
Pantoprazole	99.44 %	0.53	0.53

Acceptance criteria:

The % RSD for the sample of Cinitapride and Pantoprazole should be NMT 2.0%.

LIMIT OF DETECTION:

Limit of detection was determined based on the standard deviation of y intercepts of the regression line. The standard deviation of y intercepts obtained from the replicate measurements (n=3) was substituted for σ in the equation 3.3σ/S, and S is the mean of slope of the calibration curves.

LIMIT OF QUANTITATION:

Limit of quantitation was determined based on the standard deviation of y-intercepts of the regression line. The standard deviation of y intercepts obtained from the replicate measurements (n=3) was substituted for σ in the equation 10σ/S, and s is the mean slopes of the three calibration curves.

TheLODandLOQvalues weredetermined bythe formulae

LOD=3.3XS/mand**LOQ=10 S/m**

Where, Sisthe standard deviationoftheresponses

mismeanoftheslopesof thecalibrationcurves.

The results were given in the table.

SPECIFICITY:

Specificity is defined as the degree to which the analyte measured is due only to the analyte of the interest and not to any other substances expected to be present in the sample matrix. For determining the specificity of the method the test solution was injected to the chromatographic system. Interferences due to the presence of excipients were not traced in the chromatogram and the chromatographic parameters were not affected. Thus the selected method is specific for the determination of the marketed formulation.

ROBUSTNESS

As part of evaluation of robustness, deliberate changes were made in the flow rate and Wavelength to evaluate the impact on the method.

Effect of Variation of Flow Rate

Standard solution prepared as per the test method was injected into the chromatographic system maintaining flow rates, less flow (0.8ml/min) and more flow (1.2 ml/min).

Table: 15 Robustness Data of CIN and PAN (0.8mL/min)

S. No	CIN		PAN	
	R_t (min)	Peak area	R_t (min)	Peak area
1	5.040	8592577	5.890	96167944
2	5.057	8533424	5.893	97467968
3	5.037	8516499	5.911	97162006
4	5.041	8448044	5.880	95721698
5	5.022	8575998	5.890	96478365
6	5.048	8442165	5.896	96747880

mean	8518118	96624310
SD	62945	641938
%RSD	0.74	0.66

Table: 16 Robustness Data of CIN and PAN (1.2mL/min)

S. No	CIN		PAN	
	R_t (min)	Peak area	R_t (min)	Peak area
1	4.220	7822305	5.090	89688853
2	4.201	7738652	5.000	90397831
3	4.198	7806791	5.054	90650360
4	4.207	7744041	5.094	90106974
5	4.205	7861332	5.039	91325064
6	4.221	7876529	5.067	91038385
mean	7808275		90534578	
SD	57700		601480	
%RSD	0.74		0.66	

Effect of Variation of Wavelength:

Standard solution prepared as per the test method was injected into the chromatographic system maintaining flow rates, less wavelength 280nm, more wavelength 282nm and actual wavelength 281nm. (Fig.: 35-37).

Table: 17 Robustness Data of CIN and PAN

S. No	Peak areas			
	280nm		282nm	
	CIN	PAN	CIN	PAN
1	8002877	92620009	7938349	91486225
2	8024688	91753925	7871160	91603127
3	7973557	91980341	7968111	92478206
4	7925458	92706273	8085965	93227923
5	8008349	92781379	7901974	92562832
6	7891102	93005443	7991125	91127487
Mean	7971005	92474562	7959447	92080967
SD	52455	492826	75668	799584
%RSD	0.66	0.53	0.95	0.87

Table 18 Statistical validation Data of Robustness

Operational parameter	Mean	SD	%RSD
Cinitapride			
Flow rate +0.2mL/min	7808275	57700	0.74
Flow rate -0.2mL/min	8518118	62945	0.74
Wavelength +1nm	7959447	75668	0.95
Wavelength -1nm	7971005	52455	0.66

Pantoprazole			
Flow rate +0.2mL/min	90534578	601480	0.66
Flow rate -0.2mL/min	96624310	641938	0.66
Wavelength +1nm	92080967	799584	0.87
Wavelength -1nm	92474562	492826	0.53

RUGGEDNESS

The ruggedness of the developed analytical method was determined by analyst variation (analyst 1 and analyst 2). The results were analysed statistically and the effect of variations were estimated.

The ruggedness of the analytical method was determined by analysis of the solutions prepared by two analyst (analyst 1 and analyst 2). Appropriate aliquots of the working stock solution was transferred to a 20mL volumetric flasks and the volume was made up to the mark with the solvent to obtain a solution of concentration 7.5 µg/mL of Cinitapride and 100 µg/mL of Pantoprazole. Six replicates of this solution was prepared by analyst 1 and analyst 2. A 20 µL of this solutions was analysed injected to the chromatographic system and the chromatograms were recorded. The results were statistically validated.

Table: 19 Ruggedness Data of CIN PAN

S. No	Peak areas			
	Analyst-1		Analyst-2	
	CIN	PAN	CIN	PAN
1	8115541	92181733	8083356	91986004
2	8060085	93555226	7971160	92310031
3	7984218	92862802	7992111	92676406
4	7950042	91683225	8005965	93344454
5	8039999	92009411	7951487	91964742
6	7914530	92567000	7931125	92067487
Mean	8010736	92476566	7989201	92391521
SD	74724	671627	53433	538045
%RSD	0.93	0.73	0.67	0.58

Table 20 Statistical Validation Data of Ruggedness

Variation	Mean	SD	%RSD
Cinitapride			
Analyst 1	8010736	74724	0.93
Analyst 2	7989201	53433	0.67
Pantoprazole			
Analyst 1	92476566	671627	0.73
Analyst 2	92391521	538045	0.58

CHROMATOGRAMS

UV Spectrum:

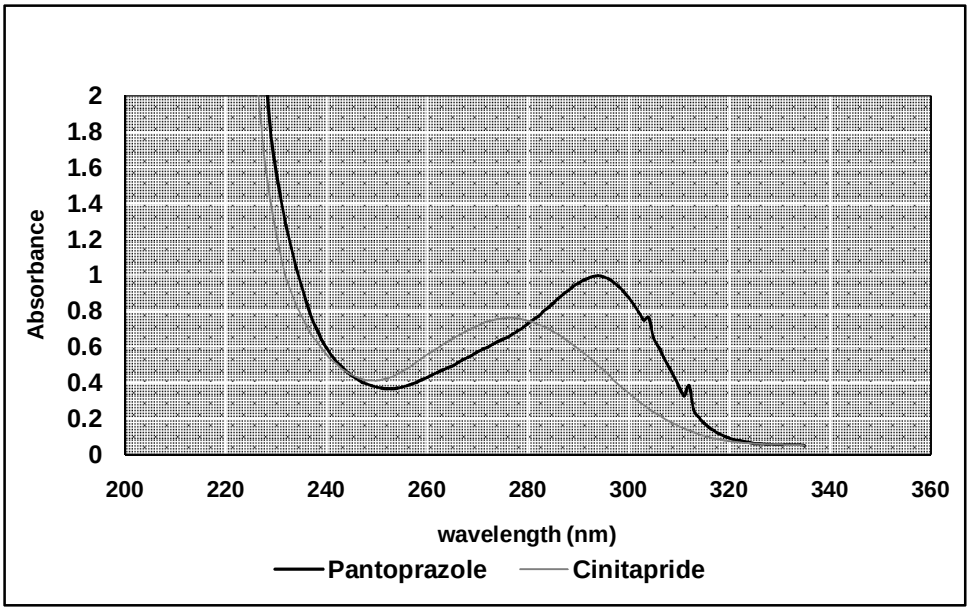


Fig.: 6

Method development trails in HPLC

Trial-1

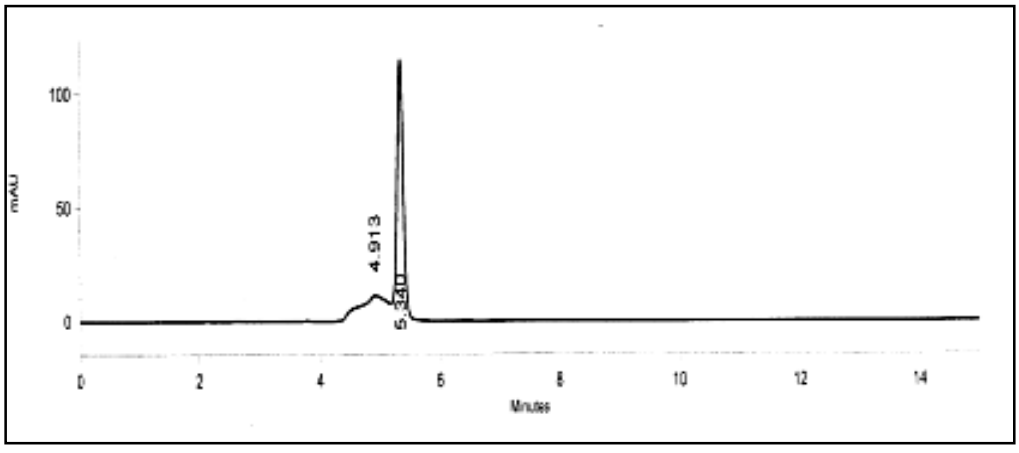


Fig.: 7

Trial-2

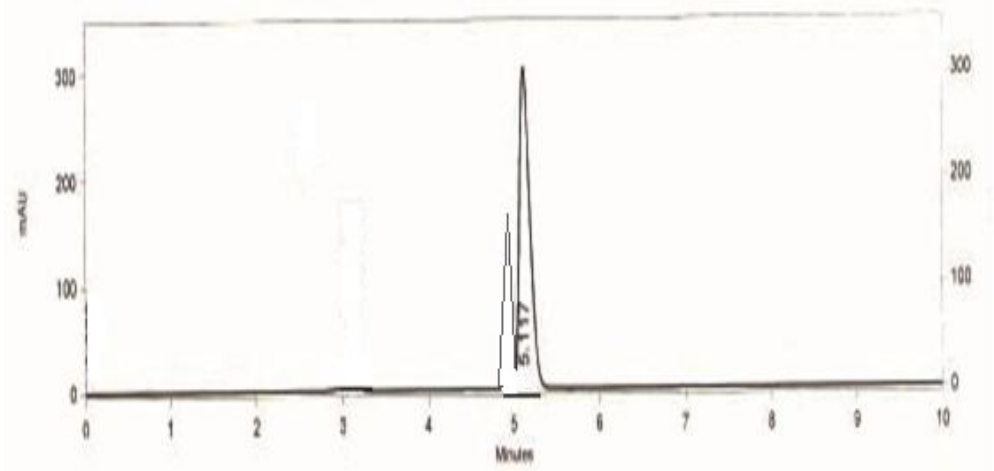


Fig.: 8

Trial-3 (Optimized Chromatogram)

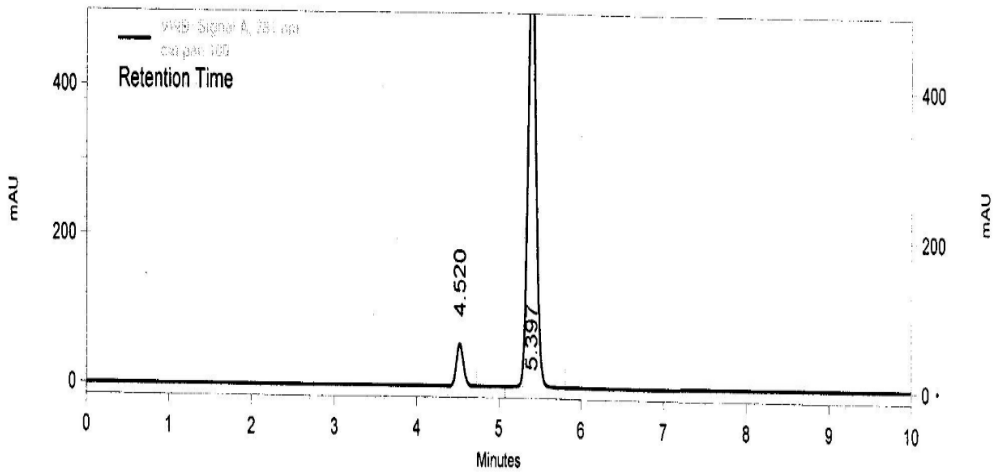


Fig.: 9

Method Validation

Blank

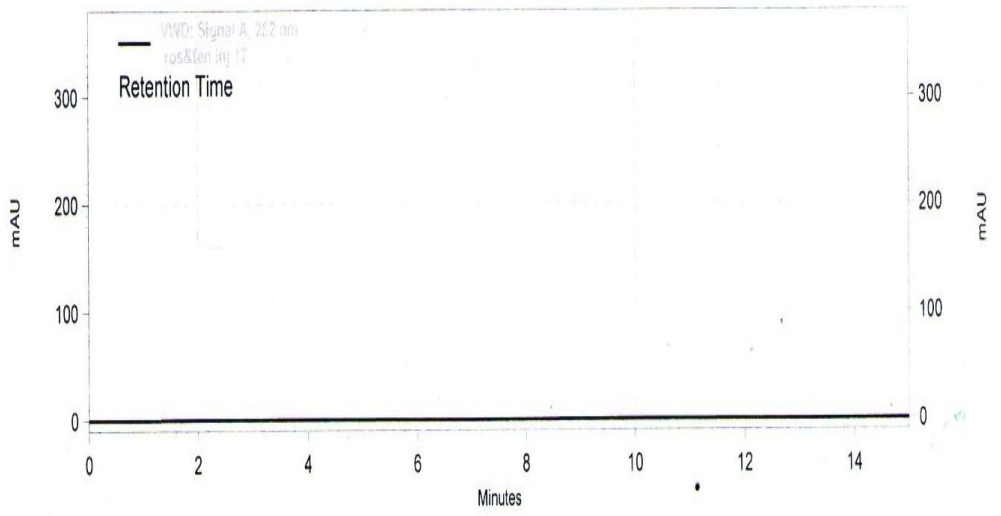


Fig.10

LINEARITY:

Level-1

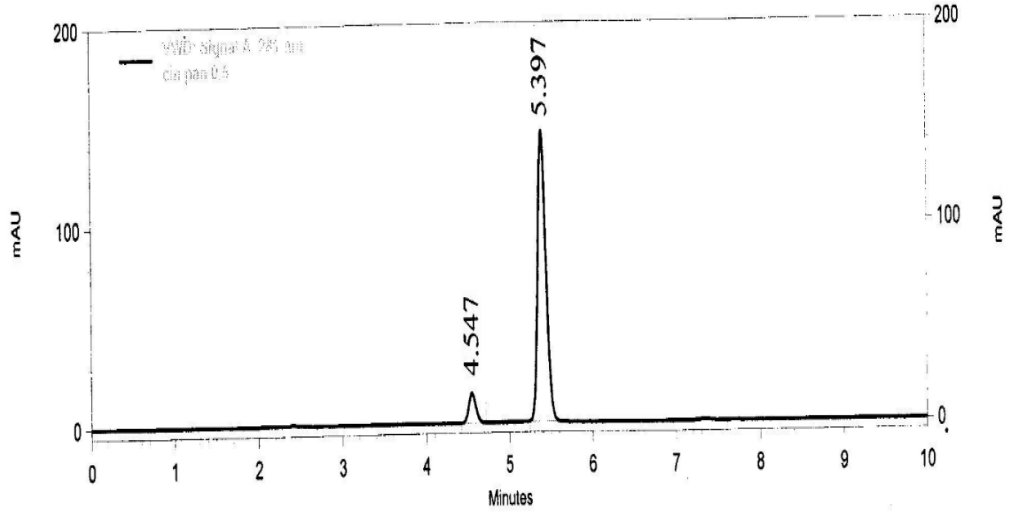


Fig.:11

Level-2

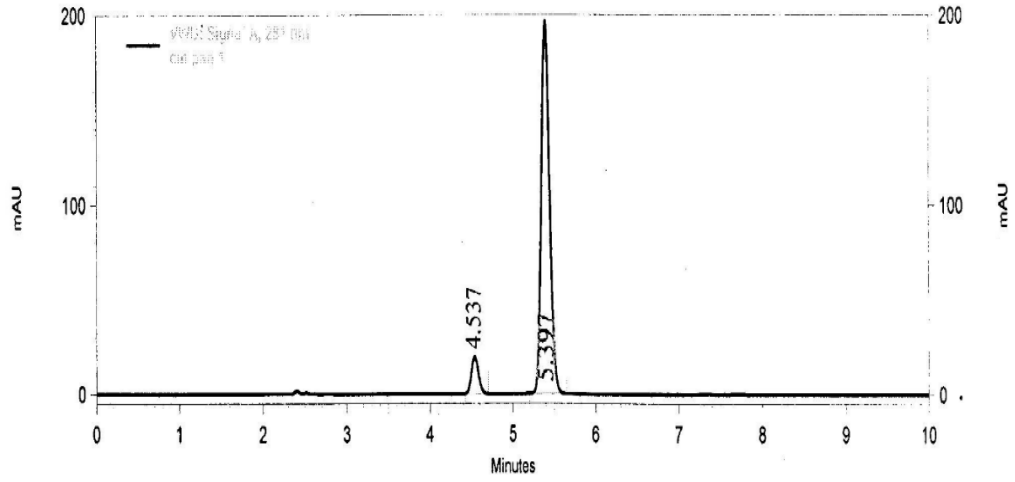


Fig.: 12

Level-3

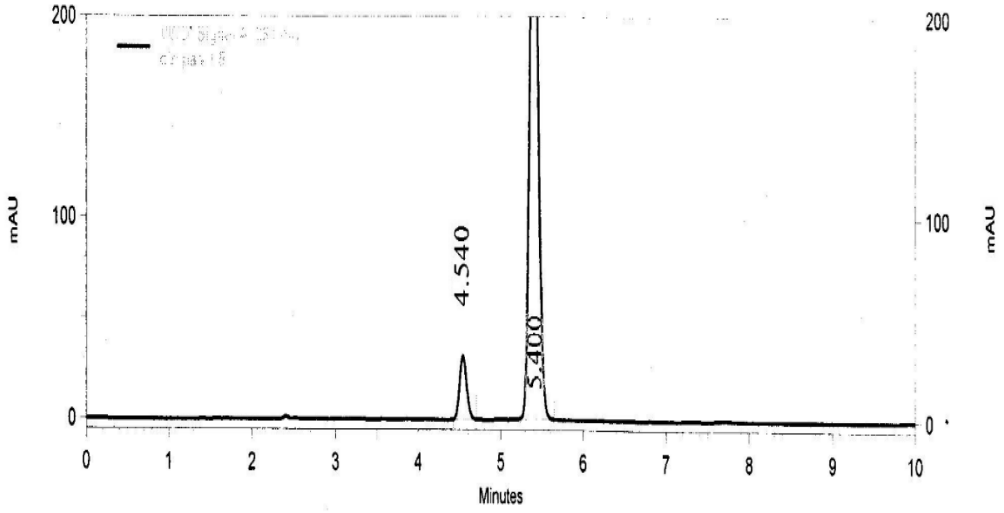


Fig.: 13

Level-4

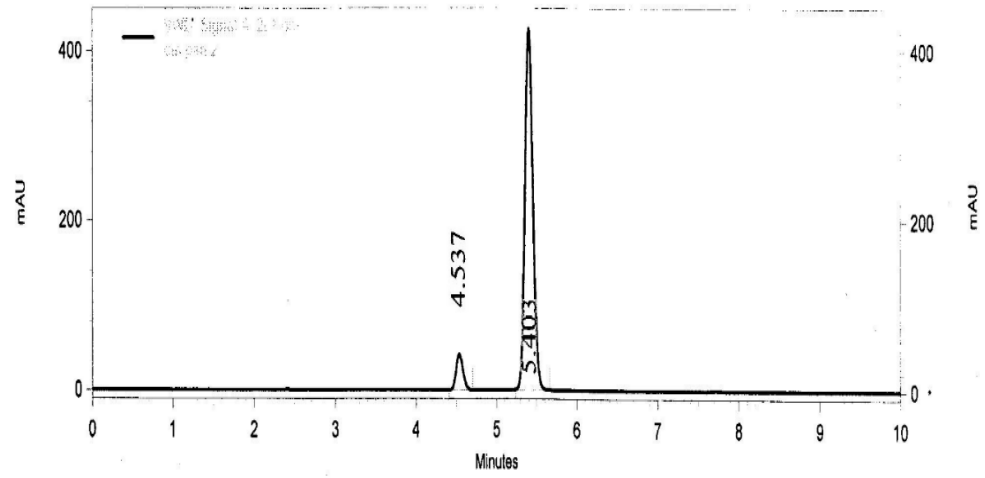


Fig.: 14

Level-5

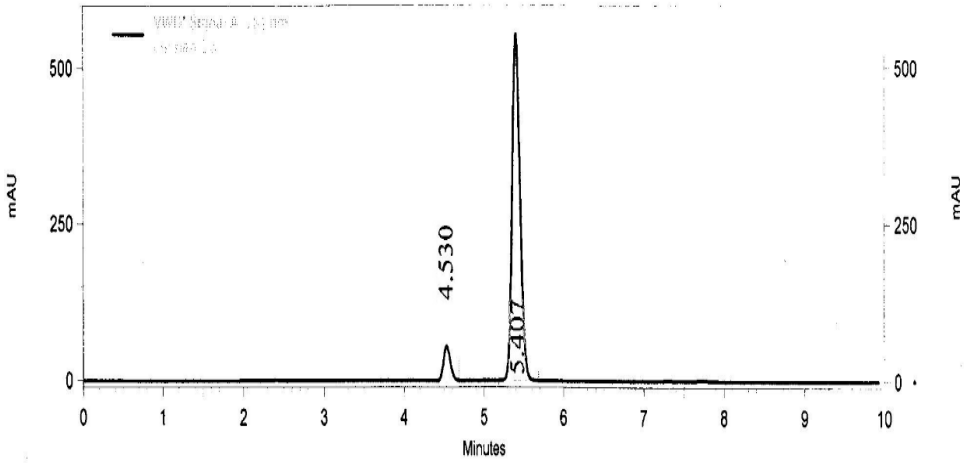


Fig.: 15

Level-6

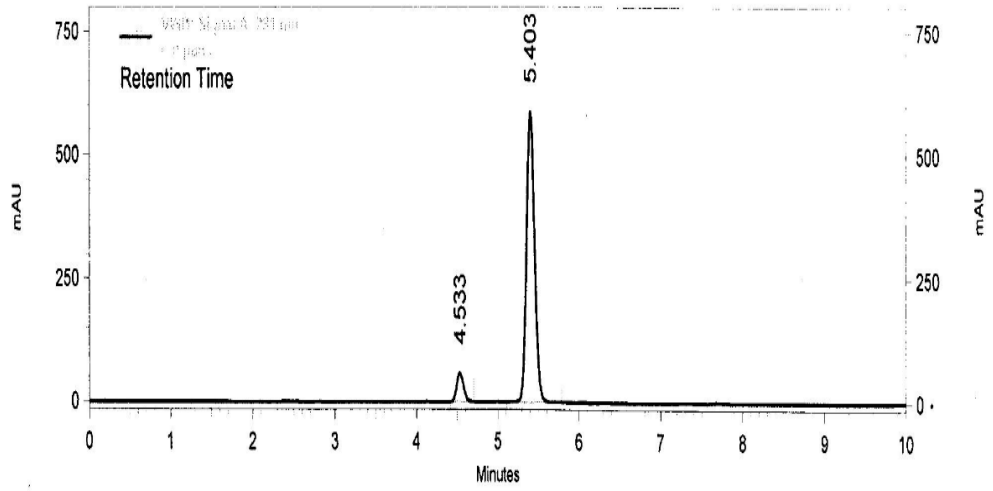


Fig.: 16

Level-7

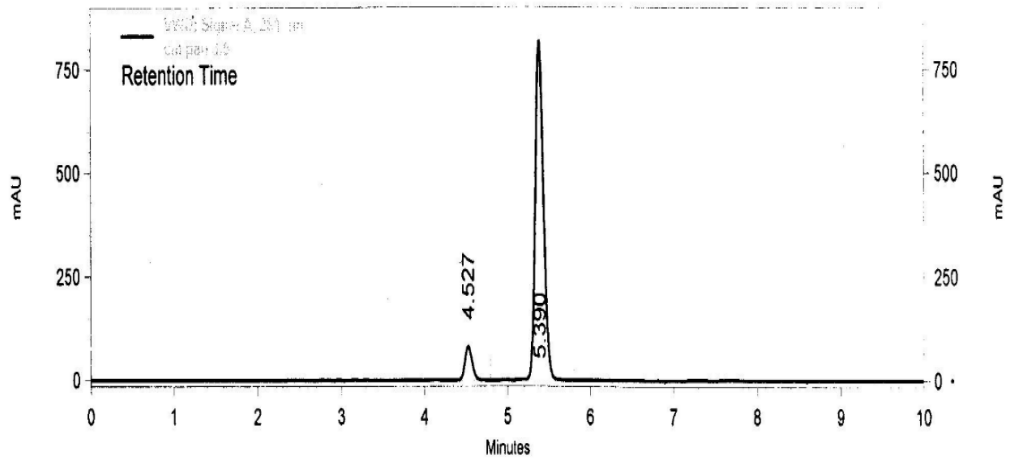


Fig.: 17

ACCURACY

Assay Chromatogram

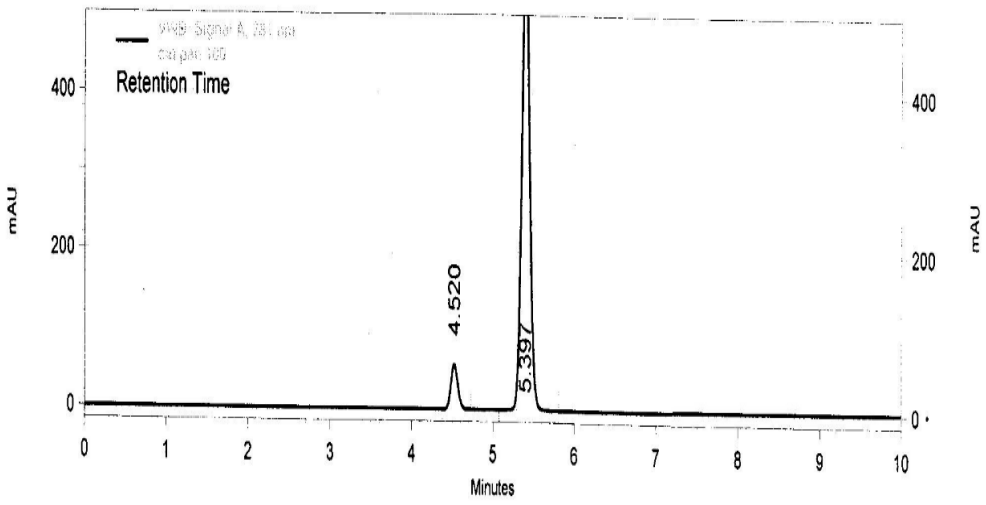


Fig.: 18

Recovery Level-1

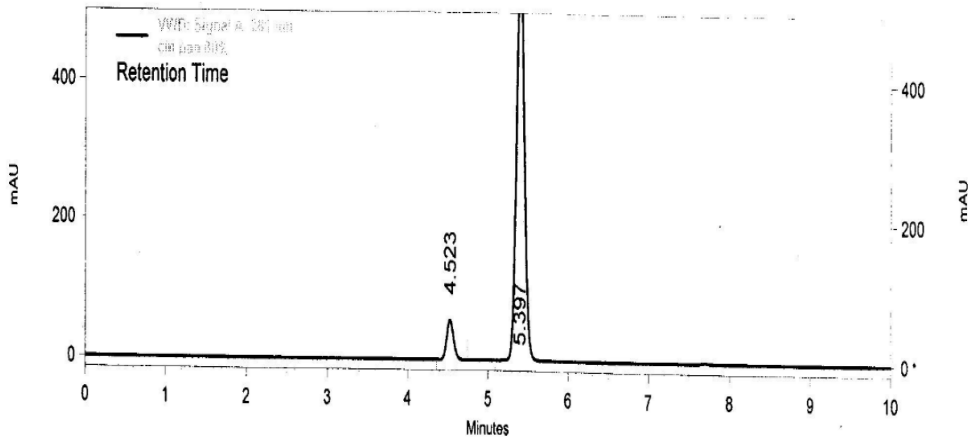


Fig.: 19

Recovery level-2

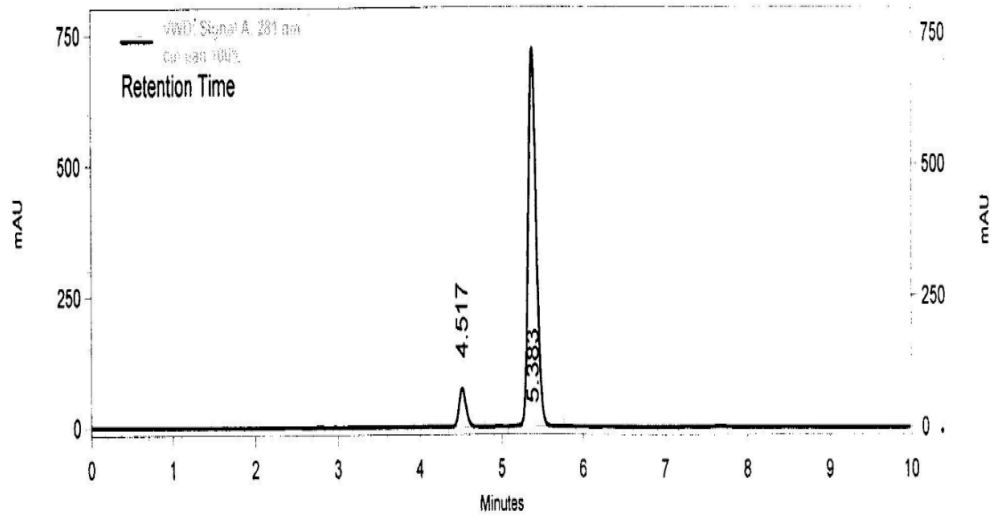


Fig.: 20

Recovery level-3

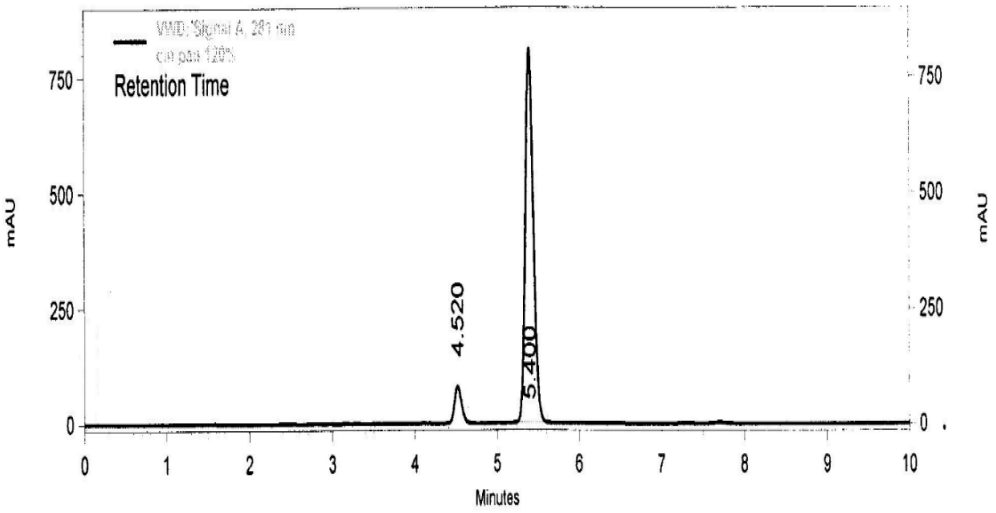


Fig.: 21

PRECISION:

Intraday Precision:

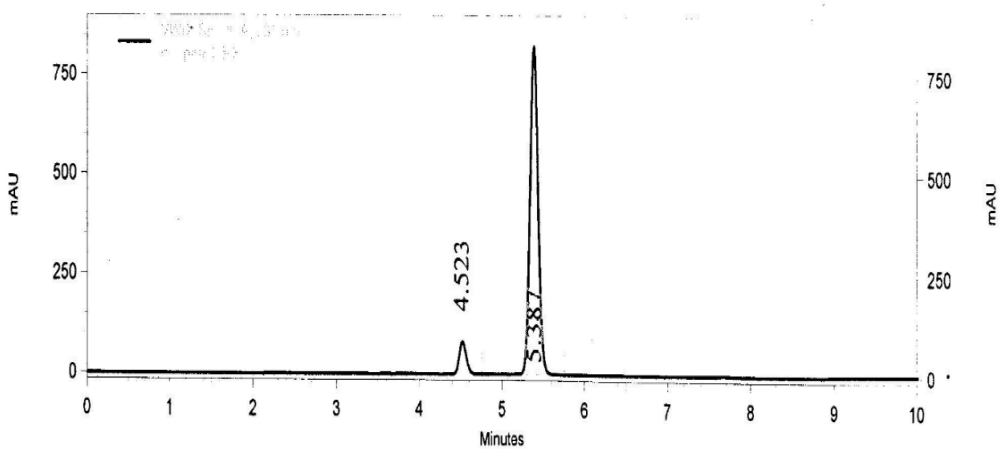


Fig.: 22

Interday Precision

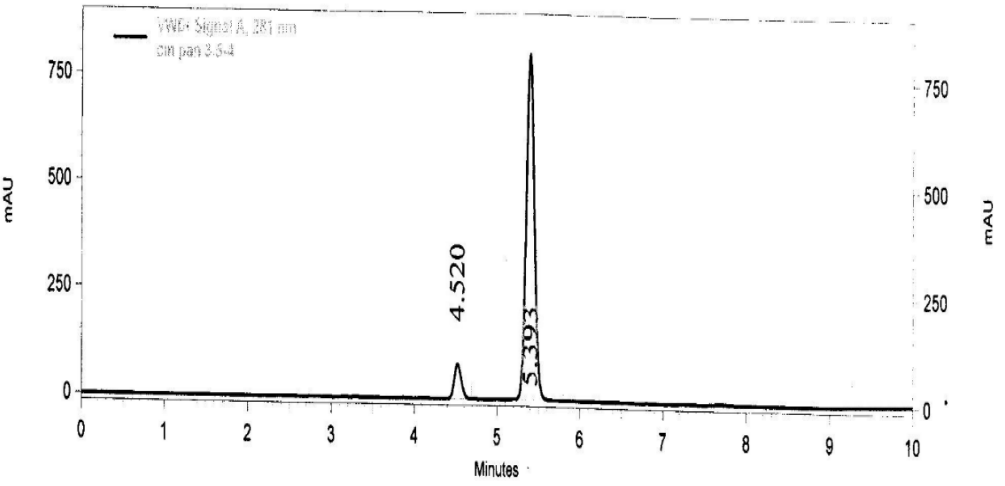


Fig.: 23

RUGGEDNESS

Analyst-1

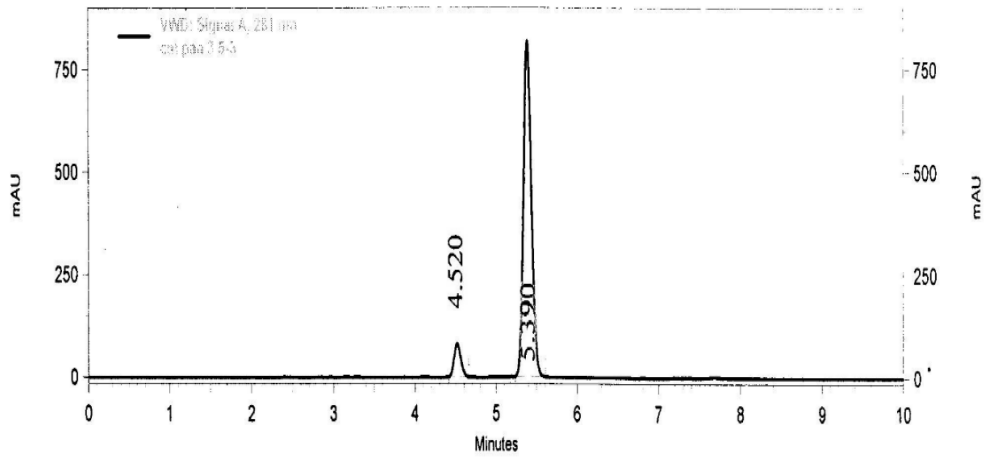


Fig.: 24

Analyst-2

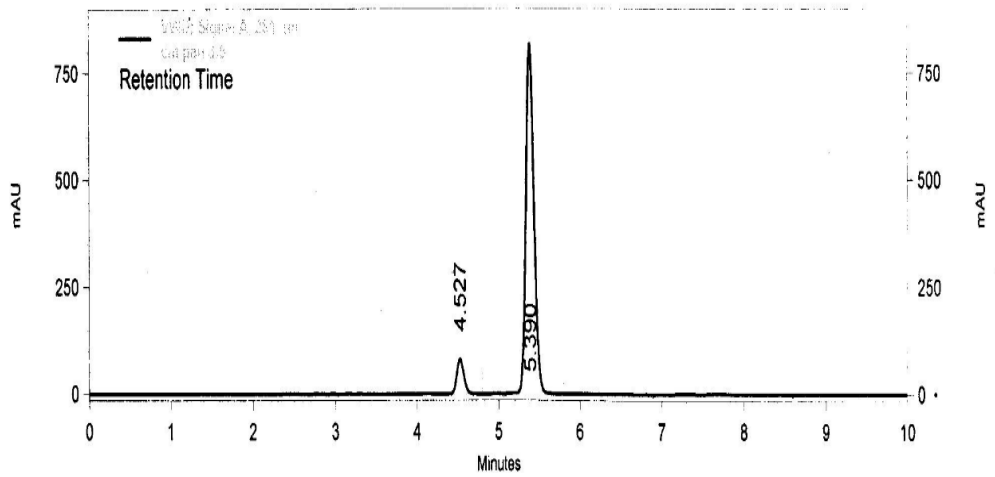


Fig.: 25

ROBUSTNESS

Wavelength at 280 nm:

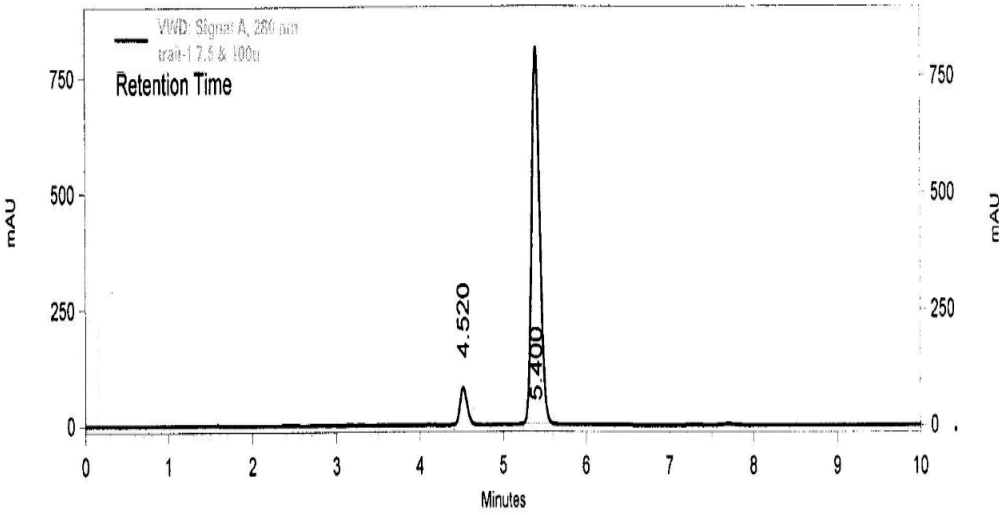


Fig.: 26

wavelength at 282 nm:

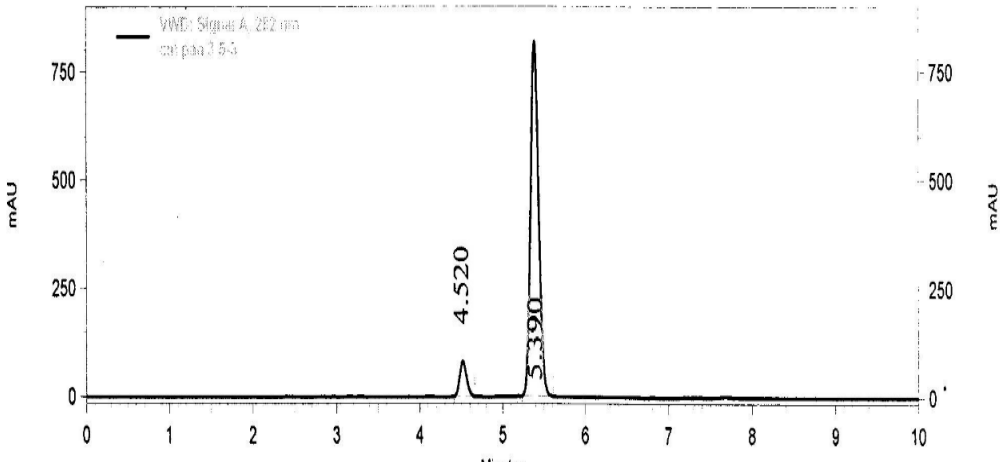


Fig.: 27

Flow rate at 0.8 mL/min:

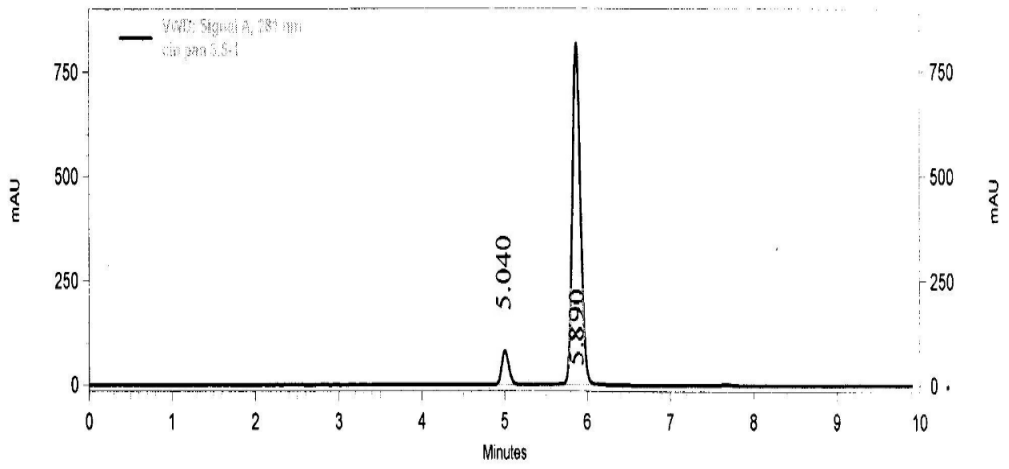


Fig.: 28

Flow rate at 1.2 mL/min:

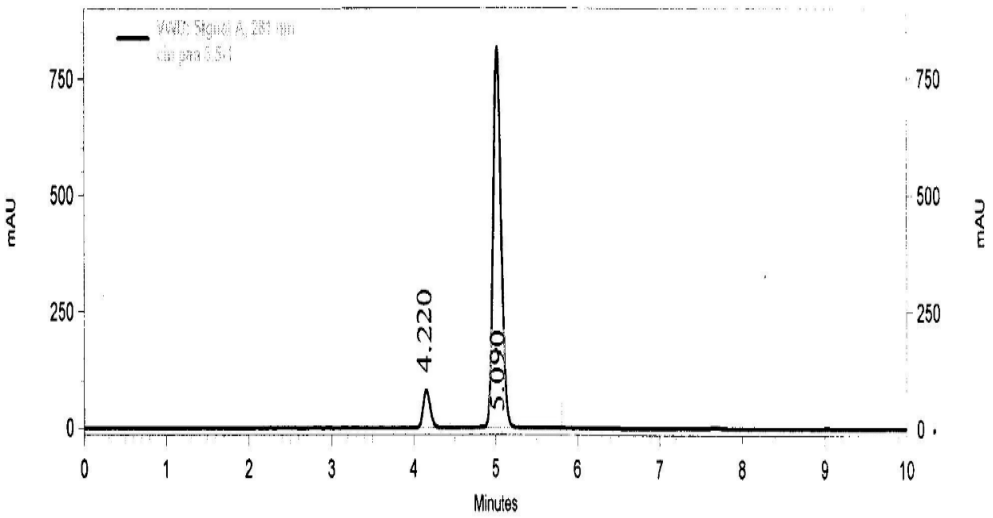


Fig.: 29

RESULT AND DISCUSSION

A simple Reverse phase high performance liquid chromatographic method has been developed and subsequently validated for Cinitapride and Pantoprazole Capsules.

The separation was carried out by using a acetonitrile and phosphate buffer (pH) 6.8 (50:50 v/v). The detection was carried out at 281 nm. The column was Zorbax ODS C_{18} (250 x 4.6mm, 5µ). The flow rate was selected as 1mL/min.

System suitability was assessed by injecting 6 replicate injections of 100% test concentration. Number of theoretical plates was more than 2000 for both the drugs and tailing factor was less than 1.5 for both Cinitapride and Pantoprazole was reported. A Resolution of greater than 2 was observed. The relative retention times of 6 replicate injections were shown in the table 1 and 2.

The peak areas corresponding to the concentration range of Cinitapride 1.5 -10.5 µg/mL and Pantoprazole 20-140 µg/mL prepared in triplicate were plotted against the respective concentrations. The calibration curves were linear in the range studied for Cinitapride and Pantoprazole, respectively, with mean correlation coefficients (n = 3) of 0.999 and higher, the representative calibration curve was shown in fig. 10. The regression analysis was given in the tables 3 and 4. The chromatogram of linearity was given in figure 11-17.

Accuracy of the method was examined by performing recovery studies by standard addition method for drug product. The recovery of the added standard to the drug product sample was calculated and it was found to be 100.64 %w/w and 100.29%w/w for Cinitapride and Pantoprazole respectively and the % RSD was less than 2 for both the drugs which indicates a good accuracy of the method. Chromatograms depicting the three recovery levels were given in figures 18-21. The results of recovery were given in the table 6-8.

The method was precise with a %RSD of less than 2 for both Cinitapride and Pantoprazole respectively. The results of intraday and inter day precision were given in the table 9,10,12 and 13. The statistical validation data is given in the tables 11 and 14. Chromatograms of precision were given in the figure 22, 23. Limit of detection of Cinitapride and Pantoprazole were 0.34 µg/mL and 3.38µg/mL respectively. Limit of quanification of Cinitapride and Pantoprazole were 1.04 µg/mL and 10.15 µg/mL respectively. LOD and LOQ values were given in the table 4.

Specificity of the chromatographic method was tested by injecting sample concentration prepared from marketed formulation. The response was compared with that obtained from the standard drug. The chromatogram confirms the presence of Cinitapride and Pantoprazole at 4.5min and 5.4min respectively without any interference. Thus the developed method was specific to Cinitapride and Pantoprazole. An optimised chromatogram with the retention times of Cinitapride and pantoprazole was given in the figure 18.

Ruggedness was carried out by change in the analyst (1 and 2), and instrument (Agilent). Solution of 100% concentration was prepared and injected in triplicate for each varied operational condition and % R.S.D was found to be less than 2. The Results were given in the table 19 & 20. The chromatograms were shown in the figure 24, 25.

Robustness was carried out by change in the flow rate (± 0.2mL/min), and variation in wavelength (± 1 nm). Solution of 100% concentration was prepared and injected in six for each varied operational condition and % R.S.D was found to be less than 2. The Results were given in the table 16-18. The chromatograms were shown in the figure 26-29.

Assay of marketed formulation; A 20 µL injection volume of test concentration containing 7.5 µg/mL Cinitapride and 100µg/mL Pantoprazole solution was injected in triplicate

to the chromatographic system and the peak response was measured. The content of each component in the formulation was estimated by comparing the peak area of the test sample with that of the peak area of the standard. The results of estimation were given in the table 5. The chromatogram was given in figure 18.

Table -21 Analytical method validation report for Cinitapride and Pantoprazole

Parameter	Results	
	Cinitapride	**Pantoprazole**
wavelength (nm)	281	
Rt (min)	4.5	5.4
Regression equation	y=754116.2x+45173.75	y=670332x-1839124
Correlation coefficient	0.9996	0.9992
Accuracy	100.64 %	100.29 %
LOD (µg/ml)	0.14	0.99
LOQ (µg/ml)	0.43	2.98
Assay	100.2 %	99.4 %
Precision (%RSD)		
Intraday precision	0.58	0.78
Inter day precision	0.65	0.53
Robustness (%RSD)		
Flow rate 1.2mL/min	0.74	0.66
Flow rate 0.8mL/min	0.74	0.66
wavelength 280nm	0.66	0.53
wavelength 282nm	0.95	0.87

Ruggedness (%RSD)		
Analyst 1	0.93	0.73
Analyst 2	0.67	0.58

CONCLUSION

A RP-HPLC method for Cinitapride and Pantoprazole were developed and validated in capsule dosage form as per ICH guidelines. The results are found to be complying with the acceptance criteria for each of the parameter.

Agilent HPLC (Open Lab software with DAD detector) with Zorbax ODS C_{18} (250X 4.6mm, 5µ) Packed Column, Injection volume of 20µL was injected and eluted with the Mobile phase (Acetonitrile : Phosphate buffer pH 6.8, in the ratio of 50:50%v/v) Which was pumped at a flow rate of 1.0 mL at 281nm. The peak of Cinitapride and Pantoprazole was found well separated at 4.5 min, 5.4 min. The developed method was validated for various parameters as per ICH guidelines like system suitability, linearity, accuracy, precision, specificity, limit of detection, limit of quantitation, ruggedness, and robustness.

Hence it is concluded that the assay method is found to be valid in terms of reliability, precision, accuracy and specificity and hence it is suitable for routine analysis as well as for stability analysis.

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